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**Nicotinic acetylcholine receptors from the parasitic nematode ascaris suum**

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# **NICOTINIC ACETYLCHOLINE RECEPTORS FROM THE PARASITIC NEMATODE *ASCARIS SUUM***

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A thesis submitted for the degree of Doctor of Philosophy  
University of Bath  
Department of Biology and Biochemistry  
November 2008

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## **Summary**

Nematodes of the genus *Ascaris* are large gastrointestinal parasites. *Ascaris lumbricoides* infects ~1 billion people globally; causing malnutrition and general morbidity, and can block the gut or bile duct causing fatal complications. *Ascaris suum* is a parasite of pigs; in addition to its veterinary significance, it can occasionally be zoonotic, and is a good model of the human parasite. One of the main classes of drugs used to treat parasitic nematode infections are the cholinergic anthelmintics, such as levamisole and pyrantel, which act as agonists of nicotinic acetylcholine receptors at the nematode neuromuscular junction. The pharmacology of the native neuromuscular nAChRs from *Ascaris suum* has previously been well described, but most detailed studies of the molecular biology of nematode nAChRs have previously been confined to the model organism *C. elegans*. The aim of the work presented here was therefore to attempt to interpret the pharmacology of the nAChRs of *Ascaris suum* in terms of the underlying molecular biology.

The genome of *Brugia malayi*, a parasite within the same phylogenetic clade as *Ascaris suum*, was searched for putative nAChR subunit sequences. The results of this search showed that parasites from this clade appear to have far fewer nAChR subunit genes than *C. elegans*, though three subunits known to be involved in levamisole sensitivity in *C. elegans*, *unc-38*, *unc-29* and *unc-63*, were present and well conserved. Full-length sequences of *unc-38* and *unc-29* were then amplified from *Ascaris suum*, though only a partial sequence of *unc-63* was obtained. An additional, novel nAChR subunit with no *C. elegans* homologue was also amplified from *Ascaris suum*.

Antisera were raised against peptides from *Ascaris suum* UNC-38, UNC-29 and UNC-63, and were used to perform indirect immunofluorescent labelling of the nAChR subunits. All three subunits were shown to be present on the membrane of muscle cells from *Ascaris suum*, and UNC-38 and UNC-29 were shown to clearly co-localise.

Expression of UNC-38 and UNC-29 in *Xenopus* oocytes yielded functional receptors which were characterised pharmacologically using two-electrode voltage-clamp electrophysiology. Both UNC-38 and UNC-29 were necessary to generate a functional receptor, which was sensitive to the agonists levamisole,

acetylcholine and nicotine, and the antagonist mecamylamine. Further experiments demonstrated that the pharmacology of the receptors could be changed by altering the stoichiometry, using different ratios of *unc-38:unc-29* RNA. Addition of more *unc-38* produced a receptor more sensitive to nicotine and oxantel, but less sensitive to levamisole and insensitive to pyrantel. Addition of more *unc-29* produced a receptor more sensitive to levamisole and pyrantel, but less sensitive to nicotine and insensitive to oxantel. The pharmacology of the receptors produced in this study by heterologous expression in oocytes resembled two distinct pharmacological subtypes of nAChR previously described from native *Ascaris suum* muscle cells.

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# **Chapter 1: Introduction**

## **1.1 Nematodes**

### **1.1.1 The diversity of nematodes**

The phylum nematoda is incredibly diverse, with estimates of number of species ranging from tens of thousands to several million (Lambshead 1993). Nematodes inhabit a diverse array of trophic niches, ranging from free-living bacteriovores found in soil and marine sediments, to parasites of plants, invertebrates and vertebrate animals (Baldwin *et al.*, 2004; Blaxter *et al.*, 1998; Lambshead, 1993). Despite superficial morphological similarities and a lack of fossil evidence, molecular analysis has greatly increased our understanding of nematode phylogeny (Blaxter *et al.*, 1998; Conway-Morris, 1981). Analysis of small subunit ribosomal RNA sequences has produced a robust phylogenetic framework for the nematodes, resolved into five monophyletic clades (Blaxter *et al.*, 1998; Dorris *et al.*, 1999). A modified version of this accepted phylogeny is shown in Figure 1.1. Interestingly, the SSU rRNA phylogeny supports multiple origins of parasitism in nematodes: plant parasitism evolved 3 times, and animal parasitism arose independantly at least 6 times during the course of nematode evolution (Blaxter *et al.*, 1998; Dorris *et al.*, 1999).

Clade I contains nematodes occupying a range of ecological niches: free-living, plant parasitic, the insect parasitic Mermithida, and the vertebrate parasite genera *Trichinella* and *Trichuris* (Blaxter *et al.*, 1998; Dorris *et al.*, 1999).

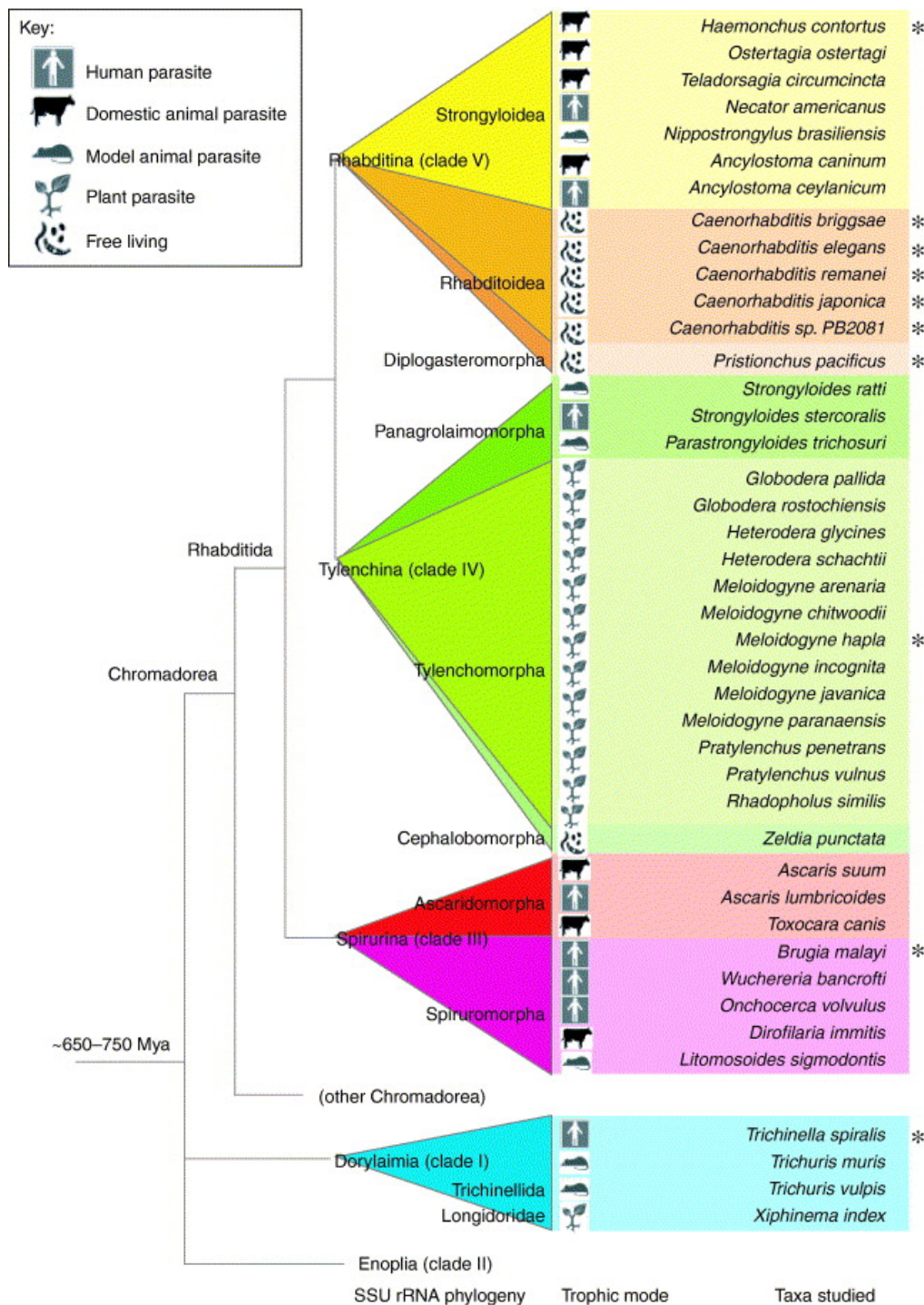
Clade II is less well studied, but is known to contain both bacteriovores and plant parasites (Blaxter *et al.*, 1998). Clade III is composed almost exclusively of animal parasites, of both invertebrates and vertebrates (Nadler *et al.*, 2007).

Basal members of this clade are often parasites of the arthropod gut, suggesting that perhaps this pre-adapted clade III nematodes to later become parasites of vertebrates (Dorris *et al.*, 1999). Clade VI contains notable plant parasites such as *Meloidogyne* and *Globodera*; also represented in this clade are the animal-parasitic Strongyloides (Blaxter *et al.*, 1998; Dorris *et al.*, 1999; Mitreva *et al.*, 2005). Clade V contains soil and sediment-dwelling groups alongside several vertebrate-parasitic taxa of medical and veterinary importance (Blaxter *et al.*,

1998). The most notable member of clade V is perhaps the free-living model organism *Caenorhabditis elegans*, the first metazoan animal for which a complete genome sequence was published (Blaxter, 1998; *C.elegans*\_Sequencing\_Consortium, 1998).

Since the completion of the *C. elegans* genome sequencing project, knowledge of the molecular biology of a diverse assemblage of nematodes has expanded: genome sequencing projects have been undertaken for other *Caenorhabditis* species, the plant parasite *Meloidogyne*, and the animal parasites *Haemonchus contortus*, *Brugia malayi* and *Trichinella spiralis* (Blaxter, 2003; Ghedin *et al.*, 2007; Mitreva *et al.*, 2005; Mitreva & Jasmer, 2006). For numerous other nematodes, EST datasets are being generated (Parkinson *et al.*, 2004; Wylie *et al.*, 2004).

In summary, the complexity of the phylum Nematoda is revealed both by disparate ecology and genetic diversity, and recent advances in gene sequencing and analysis have begun to reveal the similarities, and differences, between members of this diverse phylum.



**Figure 1.1: The phylogeny of nematodes**

This diagram is taken from Mitreva *et al.*, 2005. The five-clade phylogeny is based on SSU rRNA analysis (Blaxter *et al.*, 1998; Dorris *et al.*, 1999), and includes species for which EST datasets or genome sequence is available. The symbols to the left of each species indicate the trophic mode of the nematode, ie. which species are parasites of plants, humans or other animals, and which are free living bacteriovores.

### **1.1.2 Parasitic nematodes**

Many nematode parasites of vertebrates are of medical and veterinary importance as they adversely affect human health and animal welfare. There is palaeontological evidence that the association between vertebrates and their nematode parasites is ancient; coprolites have revealed that dinosaurs in the Cretaceous period carried ascarid parasites whose eggs are very similar to those produced by extant *Ascaris* species (Poinar & Boucot, 2006). The nematode parasites of humans also have a long-established history, with pinworm eggs (*Enterobius vermicularis*) being found in human faecal material dating from 10,000 years ago (Fry & Moore, 1969). The entire range of nematode parasites are too numerous to describe, but summarised here are notable examples of the main types of parasitic nematodes which have an impact on human health.

Soil transmitted helminths are gastrointestinal parasites prevalent in many tropical countries with poor sanitation; infection rates are estimated at approximately a billion people worldwide (Bethony *et al.*, 2006; de Silva *et al.*, 2003; WHO, 2005). This diverse collection of parasites includes the large roundworm *Ascaris lumbricoides*, the whipworm *Trichuris trichiura*, and the hookworms *Ancylostoma duodenale* and *Necator americanus*; many individuals in developing countries may be chronically infected with all three types (Bethony *et al.*, 2006). Although complications from such infections may directly cause mortality, the more widespread and insidious impact of these parasites is from long-term morbidity (Bethony *et al.*, 2006; WHO, 2005). Anaemia, malnutrition, and impaired growth and cognitive development are common sequelae of heavy helminth infections of children living in poverty (Bethony *et al.*, 2006; WHO, 2005). In addition to this, diseases such as HIV/AIDS and malaria are exacerbated by co-infection with nematode parasites (Fincham *et al.*, 2003; Le Hesran *et al.*, 2004).

More obvious pathologies are produced by infection with a sub-group of clade III nematodes, the filariae. These parasites are transmitted as microfilarial larvae by intermediate arthropod hosts, usually biting mosquitoes or blackflies, and develop within the tissues of their human or animal host. Lymphatic



filariasis, caused mainly by *Brugia malayi* and *Wuchereria bancrofti*, occurs in ~80 countries and infects ~120 million people (Wynd *et al.*, 2007). Complications of this disease cause blocked and swollen lymphatic ducts, leading to fluid accumulation and grotesque swelling of the lower limbs or male genitalia (Partono, 1987). Infection with *Onchocerca volvulus*, the causative agent of "river blindness", causes visual impairment in approximately half a million people in sub-Saharan Africa (WHO, 1995). This symptom is caused by the inflammatory immune response to microfilarial larvae entering the conjunctiva and cornea of the eye (Hall & Pearlman, 1999). The microfilariae also migrate through the skin, causing chronic and disfiguring dermatitis (Kale, 1998).

Infection with parasitic nematodes overall causes a human disease burden second only to malaria in terms of disability-adjusted life years (Mathers *et al.*, 2007). In terms of the number of years through which people must live with the debilitating effects of chronic nematode infections, and indeed the number of years of life lost through acute infections, diseases caused by parasitic nematodes rival malaria and schistosomiasis in terms of their impact on human health in tropical countries (Mathers *et al.*, 2007).

### **1.1.3 Ascaris**

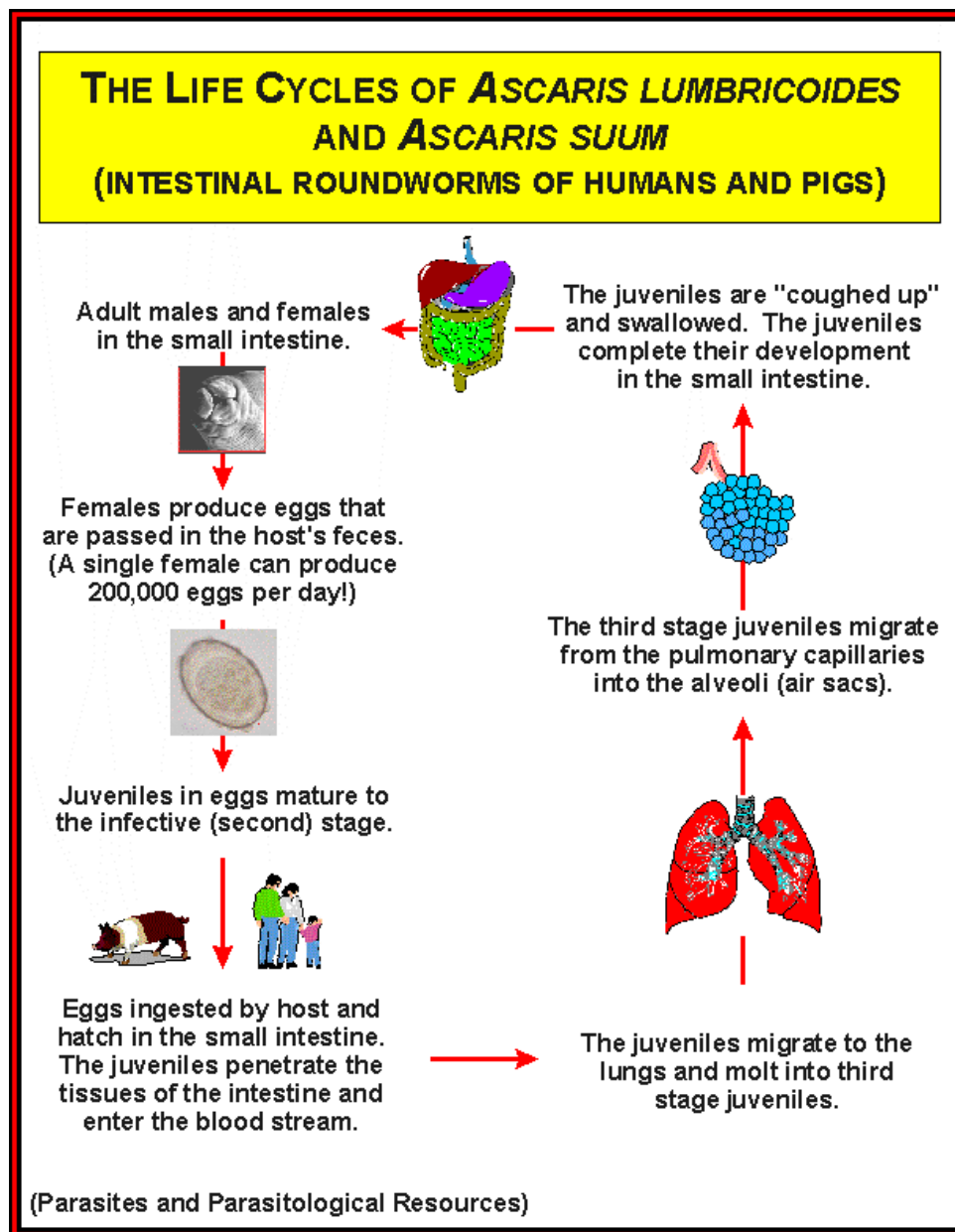
Nematodes of the genus *Ascaris* are large gastrointestinal parasites of pigs (*A. suum*) and humans (*A. lumbricoides*). Related ascarid parasites also occur in other mammals, birds and reptiles, and fossilised parasite eggs indicate that dinosaurs were also infected with an ascarid parasite, *Ascarites* (Poinar & Boucot, 2006).

*Ascaris lumbricoides* is transmitted to humans from soil contaminated with infected faecal material, so has a high prevalence in areas with poor sanitation. It is estimated that approximately 1000 million people globally are infected with *Ascaris* (Bethony *et al.*, 2006). After ingestion of embryonated eggs, the larvae hatch in the gut, penetrate the intestinal mucosa and migrate via the liver to the lungs. The pulmonary stage of *Ascaris* infection may give rise to associated symptoms such as pulmonary eosinophilia (Chitkara & Krishna, 2006). The larvae ascend the respiratory tract and are swallowed, then

subsequently develop to adults within the intestines. The lifecycle of *Ascaris* is summarised in Figure 1.2.

Considerable morbidity is associated with *Ascaris* infection, particularly in children; malnutrition leading to impaired development is a common consequence (Crompton, 1986; Hlaing, 1993). Pathologies arising from *Ascaris* infection include blockage of the pancreatic and biliary duct, and where a heavy worm burden is present intestinal occlusion may occur; such complications result in hospitalisation and may cause fatalities (Bahu, 2001; de Silva, 1997).

The pig parasite *Ascaris suum* is considered a good experimental model of human ascariasis (Boes & Helwich, 2000). There is also evidence of humans infected with *Ascaris suum*; microsatellite markers suggest that *Ascaris* populations have switched between porcine and human hosts several times during their recent evolution (Criscione *et al.*, 2007). Ascariasis may primarily be a zoonosis in communities with no endemic *Ascaris lumbricoides* and which have close contact with swine harbouring *Ascaris suum* (Criscione *et al.*, 2007; Nejsun *et al.*, 2005). In addition to its role in human disease, *Ascaris suum* is studied as a helminth of veterinary significance. Infection of farmed pigs lowers productivity and has considerable economic consequences; losses of \$155 million per year due to *Ascaris suum* infections in US swine herds have been reported (Stewart & Hale, 1988).



**Figure 1.1: The lifecycle of *Ascaris spp.***

*Ascaris suum* and *Ascaris lumbricoides* are large gastrointestinal parasites of swine and humans respectively. The eggs are soil-transmitted, ie. expelled in faecal material and transmitted to a new host by ingestion of eggs. The infective larvae hatch in the gut and undergo a complicated migration via the bloodstream to the lungs, before re-entering the gut and developing to adults in the small intestine.

## **1.2 Anthelmintics**

### **1.2.1 Anthelmintics**

Since the 1970s, 3 classes of broad-spectrum anthelmintics have been used to treat nematode infestations of veterinary and medical significance: the benzimidazoles, the macrocyclic lactones (eg. avermectins), and the imidazothiazoles (eg. levamisole) (Burg *et al.*, 1979; Thienpont *et al.*, 1966).

Benzimidazoles were originally used as antifungal agents; their mode of action is to prevent polymerisation of  $\beta$ -tubulin in the pathogenic organism (Allen & Gottlieb, 1970; Davidse & Flach, 1978). However, despite the continuing use of these compounds, resistance rapidly emerged in both fungi and nematodes, necessitating the development of alternative anthelmintics (Hastie & Georgopoulos, 1971; Theodorides *et al.*, 1970).

The macrocyclic lactones and the imidathiazoles both target cys-loop ligand-gated ion channels of nematodes; they cause paralysis of the worm, which in the case of gastrointestinal nematodes leads to their expulsion from the gut. The macrocyclic lactones also have the advantage of acting on ion channels of arthropods, so are also effective against ectoparasites and insect disease vectors (Goudie *et al.*, 1993; Mellin *et al.*, 1983). The targets of the avermectins are post-synaptic ligand-gated chloride channels; initially this was thought to be mainly GABA receptors, but later studies discovered that the glutamate-gated chloride channels are the main target in nematodes (Holden-Dye & Walker, 1990; Wolstenholme & Rogers, 2005; Yates *et al.*, 2003; Yates & Wolstenholme, 2004). The avermectins bind with high affinity to nematode chloride channels and open them irreversibly, leading to an effective inhibition of muscle contraction and therefore a flaccid paralysis of the parasite (Wolstenholme & Rogers, 2005).

The imidazothiazoles target excitatory nAChRs at the nematode neuromuscular junction, causing prolonged muscle contraction and a spastic paralysis in the nematode (Aceves *et al.*, 1970). The effects of levamisole on nematode neuromuscular nAChRs are discussed further in section 1.4.1.

More recently, two new classes of anthelmintic drugs have been developed, with the advantage of being effective against nematodes resistant to the other classes of anthelmintic: these are the cyclo-octodepsipeptide emodepside, and

the aminoacetonitrile derivatives (AADs) (Harder *et al.*, 2003; Kaminsky *et al.*, 2008). Emodepside has been shown to bind to latrophilin and inhibit pharyngeal muscle in *C. elegans*, but its main mode of action is thought to be via calcium-activated potassium channels, where it acts to cause paralysis (Guest *et al.*, 2007; Harder *et al.*, 2005; Harder *et al.*, 2003). The AADs, like the imidazothiazoles, target nematode nAChRs, but in the case of AADs, a different subtype of nAChR is targeted. This nAChR contains the subunit ACR-23, belonging to a poorly-characterised but nematode-specific group of neuronal nAChR subunits (Kaminsky *et al.*, 2008; Mongan *et al.*, 1998; Yassin *et al.*, 2001). The AAD compounds have been shown to be effective against levamisole-resistant nematodes, confirming that they target a different nAChR subtype (Kaminsky, Ducray *et al.* 2008).

### **1.2.2 Resistance**

Drug resistance in nematodes is defined as occurring when a greater frequency of individuals in the parasite population are no longer affected by the usual efficacious dose of anthelmintic due to an inherited characteristic (Prichard *et al.*, 1980). The main mechanisms by which resistance develops are changes in the drug target site or upregulation of mechanisms which detoxify or remove the drug (Wolstenholme *et al.*, 2004). In a veterinary context, widespread and indiscriminate use of anthelmintics has led to selection for resistance alleles in the nematode parasites of a range of hosts. Drug resistance is a widespread problem in sheep and other small ruminants, and also occurs in cattle and horses (Jackson & Coop, 2000; Kuzmina & Kharchenko, 2008; Loveridge *et al.*, 2003). Resistance has developed to all 3 of the most widely used anthelmintics, and in some cases, parasite populations may be resistant to all 3 drug types, presenting serious problems for parasite control and animal welfare (Bartley *et al.*, 2004; Coles *et al.*, 1996). In a medical context, there have been reports of potential drug resistance in the causative agent of river blindness, *Onchocerca volvulus* (Awadzi *et al.*, 2004). With the advent of mass chemotherapy programmes to treat parasitic diseases of humans, it is likely that more cases of drug resistance will emerge.

Benzimidazole resistance is probably the best understood in terms of the underlying mechanisms; its history as an antifungal agent, to which fungi could become resistant, set a precedent for this (Allen & Gottlieb, 1970; Theodorides *et al.*, 1970). Mutations in  $\beta$ -tubulin which prevent the drug binding have been found in benzimidazole resistant *Haemonchus contortus*, *Cooperia onchophora*, and *Teladorsagia circumcincta*; this is therefore likely to be a major resistance mechanism (Kwa *et al.*, 1994; Silvestre & Cabaret, 2002). However, it is worth noting that additional non-specific mechanisms such as upregulation of P-glycoprotein efflux pumps could also contribute to benzimidazole resistance; this is also likely to be a factor in certain cases of avermectin resistance (Blackhall *et al.*, 1998; Blackhall *et al.*, 2008; Kerboeuf *et al.*, 2003).

Aside from the involvement of P-glycoproteins, no widespread mechanism of resistance to the avermectins has been clearly demonstrated. Mutations in glutamate-gated chloride channel genes confer reduced ivermectin sensitivity to *C. elegans* and to parasite glutamate-gated chloride channel genes expressed in the laboratory, but evidence of such mutations in field populations of parasites remains scarce (Dent *et al.*, 2000; McCavera *et al.*, 2007).

Levamisole resistance in parasites remains equally hard to define on a molecular level, although numerous studies have shown that mutating certain nAChR subunit genes in *C. elegans* affect levamisole sensitivity (Culetto *et al.*, 2004; Fleming *et al.*, 1997; Lewis *et al.*, 1980). It has been shown that loss of a levamisole-sensitive nAChR subtype occurs in levamisole-resistant *Oesophagostomum dentatum*, but the genetic changes behind this remain unknown (Martin & Robertson, 2000; Robertson *et al.*, 1999). A paucity of information regarding the molecular biology of levamisole-receptors in parasites has hindered the search for any potential target site mutations associated with resistance.

## **1.3 Cys-loop ligand gated ion channels**

### **1.3.1 Diversity and general structure of cys-loop LGICs**

The cys-loop ligand gated ion channels are pentameric proteins; each subunit is composed of an N-terminal ligand binding domain, and 4 transmembrane regions which form the ion channel (Sine & Engel, 2006). The term "cys-loop" refers to a characteristic closed loop motif formed by a disulphide bond between 2 cysteine residues in the N-terminal part of the protein. The native ligand is usually a neurotransmitter, and ligand binding causes a conformational change which induces the channel to open (Lester *et al.*, 2004). The second transmembrane regions line the ion channel pore, and charged residues in this region confer cation or anion selectivity (Galzi *et al.*, 1992). The structure of the ligand binding domain has been well described, due to a resolved crystal structure of the acetylcholine binding protein from *Lymnaea stagnalis* (Brejc *et al.*, 2002). This protein is soluble and lacks transmembrane domains, but has the pentameric conformation and ligand binding loops characteristic of the cys-loop LGICs (Brejc *et al.*, 2002).

The cys-loop LGIC superfamily is notable for its diversity, and for the remarkable degree of conservation of the key motifs among different organisms. Identification of LGICs from prokaryotes suggest these are the evolutionary precursors of eukaryotic cys-loop LGICs; the original function of these channels is thought to be chemotaxis (Tasneem *et al.*, 2005). A molecular clock approach (ie. analysis of mutation accumulation rates) dates the origin of the LGICs at approximately 2500 million years ago (Ortells & Lunt, 1995). Certain members of the cys-loop LGIC superfamily are conserved in both vertebrates and invertebrates, which implies they were present in the last bilaterian ancestor (Dent, 2006; Ortells & Lunt, 1995). These include the acetylcholine and serotonin gated cation channels, and the GABA and glycine gated anion channels (Ortells & Lunt, 1995). Subsequent to the evolutionary divergence of the different animal phyla, the cys-loop LGIC families underwent lineage-specific expansion events and losses resulting in the diversity of

channel genes present in extant taxa (Dent, 2006). Perhaps surprisingly, the vertebrates retain only a relatively small cys-loop LGIC family (Dent, 2006). In contrast, the nematode *C. elegans* possesses the largest described cys-loop LGIC gene family, with 102 members; a large sub-set of these genes form an "orphan group" for which no ligand or function has yet been identified (Brown *et al.*, 2006; Jones & Sattelle, 2008). Invertebrates also possess acetylcholine and serotonin gated anion channels, glutamate-gated cys-loop ligand-gated anion channels (distinct from the vertebrate tetrameric glutamate receptors) and also excitatory GABA-gated cation channels (Beg & Jorgensen, 2003; Putrenko *et al.*, 2005; Ranganathan *et al.*, 2000; Yates & Wolstenholme, 2004). It has been proposed that animals with a very simple nervous system (such as the 302 neurons of *C. elegans*) may utilize a large cys-loop LGIC family to enhance their neurotransmission repertoire; more complex neural circuits may reduce the need for such molecular diversity in signalling proteins (Littleton & Ganetzky, 2000).

### **1.3.2 Nicotinic acetylcholine receptors**

Nicotinic acetylcholine receptors are the most well characterised of the cys-loop ligand-gated ion channels. Like all members of the LGICs, the nAChRs are pentameric, composed of five subunits arranged around the ion channel pore. Evidence for the pentameric nature of nAChRs was first provided by studies of material from the electric ray *Torpedo marmorata*, the electric organs of which are a rich source of nAChRs (Brisson & Unwin, 1985). Tubular crystals of nAChRs from *Torpedo* have provided the basis for many structural studies of nAChRs (Unwin, 1998; Unwin, 2005). Each subunit of the nAChR is composed of 3 distinct domains. The N-terminal region of the protein is extracellular and protrudes into the synaptic cleft; this is the ligand-binding domain, and also bears the cys-loop characteristic of this LGIC family (Arias, 1997). The transmembrane domain forms the ion channel and is composed of 4 membrane-spanning components, TM1-TM4, believed to be hydrophobic alpha-helices. The ligand-binding and ion-channel domains and their role in nAChR function are described in more detail in sections 1.3.3 and 1.3.4. The cytoplasmic region,

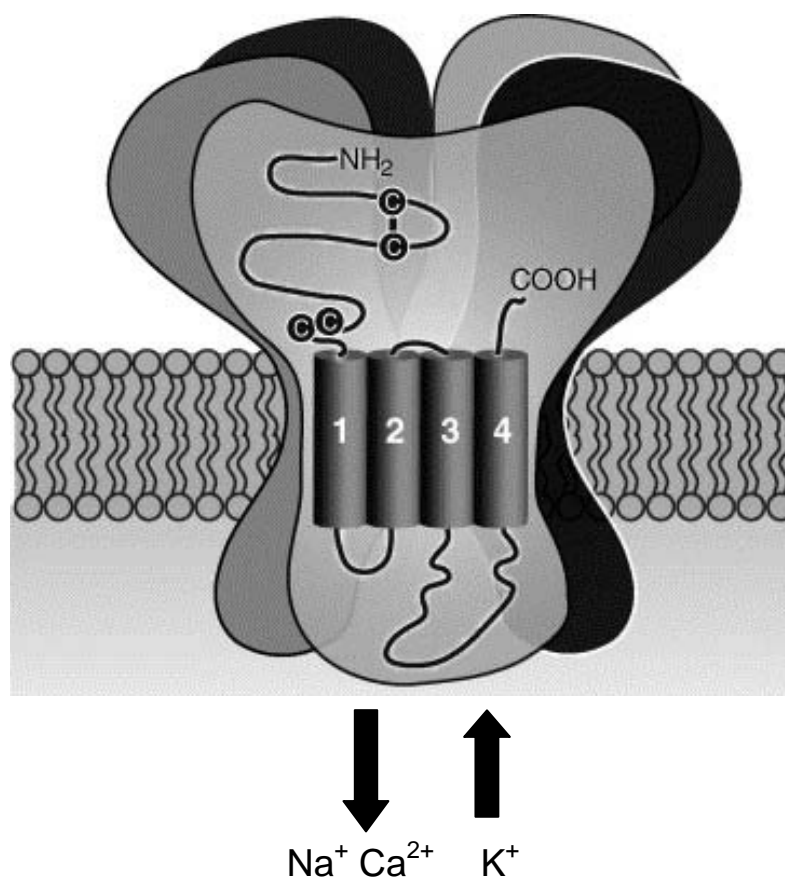


particularly the long cytoplasmic loop between TM3 and TM4, bears numerous phosphorylation sites which play a role in modulating receptor sensitivity (Huganir & Greengard, 1990). The cytoplasmic region also interacts with proteins involved in nAChR clustering (Phillips *et al.*, 1991).

The general structure of the nAChR is shown in Figure 1.3.

**Figure 1.2: Structure of the nicotinic acetylcholine receptor**

This diagram is adapted from Brown *et al.* 2006. The nAChR is a pentamer composed of five subunits, though the structure is only shown here for one subunit. The N-terminal regions of the subunits are extracellular and form the acetylcholine binding sites, and also bear the cys-loop characteristic of this receptor family of ligand gated ion channels. The transmembrane domains, here labelled 1-4, form the ion channel, which opens as a result of conformational changes induced by ligand binding.



### **1.3.3 nAChR subunit composition and stoichiometry**

Vertebrates possess 17 nAChR subunits, including  $\alpha 1$ - $\alpha 10$  (though  $\alpha 8$  appears to be specific to birds, and  $\alpha 5$  lacks the loop C tyrosine residue characteristic of  $\alpha$  subunits described below, and in section 1.3.4),  $\beta 1$ - $\beta 4$ ,  $\gamma$ ,  $\delta$ , and  $\epsilon$  (Millar & Gotti, 2008). The distinction between  $\alpha$  and non- $\alpha$  subunits was initially based on the presence of two adjacent cysteine residues involved in ligand binding first described from the *Torpedo*  $\alpha 1$  subunit cDNA (Noda *et al.*, 1982). It should be noted that the presence of these two residues alone is not necessarily an indication that a subunit can form the principal components of a ligand binding site, and later work suggests that the definition of an  $\alpha$  subunit should also include an important tyrosine residue, ie. the defining motif would be Y-X-CC (Abramson & Taylor, 1990). The role of these and other amino acids in nAChR ligand binding is described further in section 1.3.3.

Nicotinic acetylcholine receptors may be homomeric, composed of only one type of subunit, or heteromeric, composed of 2-4 different subunit types. The nAChR of the *Torpedo* electric organ has the same subunit composition as most vertebrate muscle nAChRs, being composed of two  $\alpha 1$  subunits, and also a  $\gamma$ ,  $\delta$  and  $\beta 1$  subunit (Einarson *et al.*, 1982; Raftery *et al.*, 1976), though in mammalian muscle an  $\epsilon$  subunit replaces  $\gamma$  soon after birth (Witzemann *et al.*, 1989). Homomeric receptors include human neuronal  $\alpha 7$ , and the avian subunit  $\alpha 8$  (Couturier *et al.*, 1990; Gerzanich *et al.*, 1994; Peng *et al.*, 1994). Most other receptors assemble from two or three different subunit types, and variations in subunit composition and stoichiometry can produce a diverse array of receptor subtypes with different calcium permeabilities and pharmacological properties (Millar & Gotti, 2008). An example of how changes to receptor stoichiometry can generate diversity in the pharmacology and channel properties of nAChRs is provided by  $\alpha 4\beta 2$  receptors, which are abundant in the mammalian brain. Receptors with the composition  $(\alpha 4)_2(\beta 2)_3$  have different agonist affinity than receptors with the  $(\alpha 4)_3(\beta 2)_2$  stoichiometry, and addition of a third type of subunit to give (for example)  $(\alpha 4)_2(\beta 2)_2\alpha 5$  further modifies receptor properties (Kuryatov *et al.*, 2008; Moroni & Bermudez, 2006; Moroni *et al.*, 2006).

#### **1.3.4 The ligand binding domain of nAChRs**

Evidence that the extracellular N-terminal domain bears the nAChR ligand binding sites has been provided by photoaffinity labelling or radioisotope labelling of the binding sites using appropriately modified agonist or competitive antagonist compounds (Changeux *et al.*, 1967; Chiara & Cohen, 1997). The contribution of the labelled sites to ligand binding has also been confirmed by mutation of these candidate amino acids and electrophysiological characterisation of the resulting recombinant receptors (Galzi *et al.*, 1990; Mishina *et al.*, 1985; Sine *et al.*, 1994). Though it is beyond the scope of this introduction to describe in detail the extensive body of work contributing to our knowledge of nAChR ligand binding, this has been comprehensively reviewed by Arias (Arias, 1997; Arias, 2000). Presented here is a summary of the nAChR components believed to make important contributions to agonist and non-competitive antagonist binding; this is also shown in Figure 1.3.2.

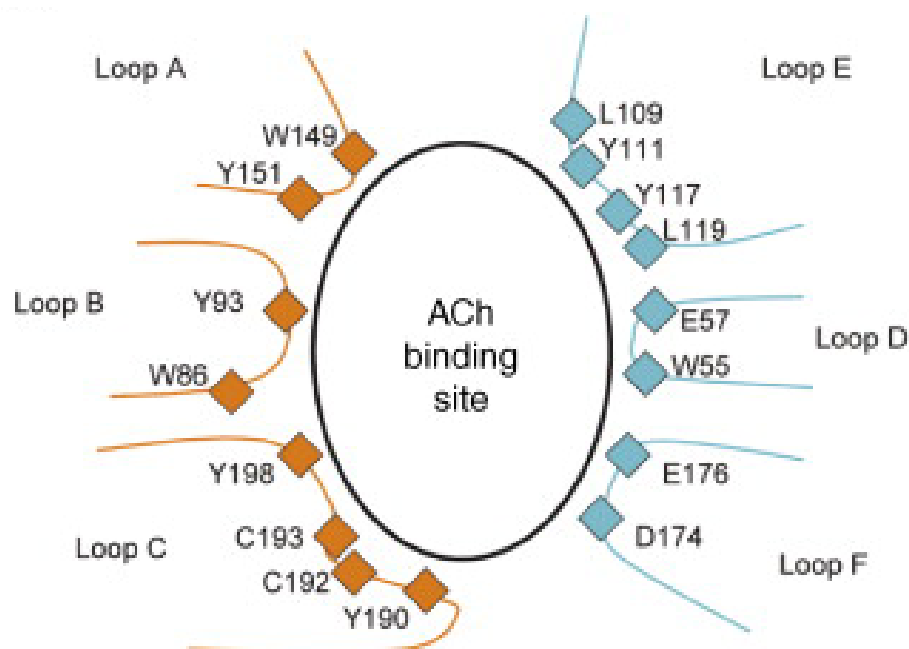
Initial photoaffinity labelling experiments showed that the  $\alpha$  subunits were the primary contributor to ligand binding (Changeux *et al.*, 1967). It has since been confirmed that several amino acids characteristic of  $\alpha$  subunits play a key role in ligand binding: in the  $\alpha 1$  subunit, these are Y190, C192, C193 and Y198, which comprise ligand binding loop C (Kao & Karlin, 1986; Mishina *et al.*, 1985; Sine *et al.*, 1994). Two additional loops from the  $\alpha$  subunits have also been found to contribute residues involved in ligand binding: loop A provides Y93 and loop B provides W149 in the Torpedo  $\alpha 1$  subunit; these residues are also conserved in  $\alpha 7$ , occurring at positions 92 and 148 (Galzi *et al.*, 1991a; Galzi *et al.*, 1990; Galzi *et al.*, 1991b). The aromatic groups provided by the ligand binding residues has been shown to be of particular importance when binding ligands with quaternary ammonium groups (Sine *et al.*, 1994).

Although the principal components of ligand binding are contributed by  $\alpha$  subunits, additional components involved in ligand binding are provided by the adjacent subunit, so that the ligand binding site is actually located at the interface between subunits; in the absence of non-alpha subunits, the muscle  $\alpha 1$  subunit does not bind agonist or d-tubocurarine (Blount & Merlie, 1989; Kurosaki *et al.*, 1987). In homomeric receptors these additional binding motifs are also present on the  $\alpha$  subunit, but in heteromeric receptors they are provided

by non- $\alpha$  subunits. These ligand binding regions are termed loops D, E and F; loop D includes a tryptophan residue at position 55 of the vertebrate muscle  $\gamma$  subunit or position 57 of the  $\delta$  subunit which is also conserved in the  $\alpha 7$  receptor at position 54, and has been labelled with both d-tubocurarine and nicotine (Chiara & Cohen, 1997; Chiara *et al.*, 1998; Corringer *et al.*, 1995). While conserved residues on the complementary binding loops may confer similarities in agonist and antagonist affinities, variations between subunits in the amino acids contributing to ligand binding can also lead to pharmacological differences. In the vertebrate muscle nAChR, the two binding sites in the same receptor have different properties, with the  $\alpha\gamma$  binding site showing a low affinity for ACh but a high affinity for d-tubocurarine, and the  $\alpha\delta$  binding site having a higher affinity for ACh but a lower affinity for d-tubocurarine (Blount & Merlie, 1989; Pedersen & Cohen, 1990). An additional example of the pharmacological properties which may be conferred on a receptor by the complementary binding components is the dependence of neonicotinoid insecticide sensitivity on residues present in loop D and loop F (Shimomura *et al.*, 2003).

**Figure 1.3: The amino acid residues of the nAChR involved in agonist binding (Changeux & Taly, 2008)**

Diagram taken from Changeux & Taly, 2008, showing the residues involved in the binding of acetylcholine. This diagram depicts the ligand binding residues from the  $\alpha 1$  and  $\gamma$  subunits from the electric organ of *Torpedo californica*. Principal binding loops A, B and C are present the  $\alpha$  subunit, with complementary binding loops D, E and F contributed by the adjacent subunit. Evidence for the involvement of these residues in ACh binding has been provided by both biochemical labelling and mutagenesis studies.



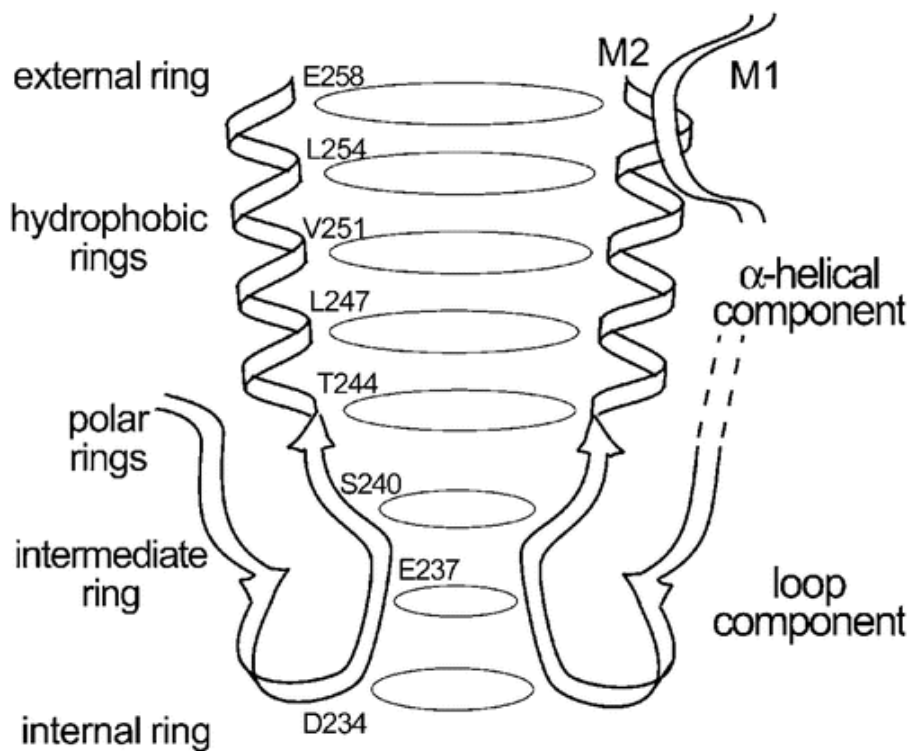
### **1.3.5 The nAChR ion channel**

On binding of ligands to the extracellular domain of the nAChR, the protein undergoes a conformational change which causes a rotational movement and consequently opens the ion channel (Sansom, 1995). This has been inferred from comparison of the structure of the *Torpedo* nAChR in the open and closed states, and the conformational change responsible for channel opening has also been modelled (Taly *et al.*, 2005; Unwin, 1993; Unwin, 1995).

The ion channel domain is formed from the transmembrane regions of the nAChR, and the second transmembrane region of each subunit forms the pore of the ion channel (Imoto *et al.*, 1986). There is also evidence that residues at the N-terminal region of the first transmembrane domain may also contribute to formation of the channel pore (Zhang & Karlin, 1997). Most of the evidence supporting the involvement of these regions in ion channel function has been provided by selective mutation of the amino acids in these regions, then heterologous expression of the resulting subunits (Akabas *et al.*, 1994; Mishina *et al.*, 1985). The ion channel of the nAChR is permeable to cations, and this specificity is conferred by three rings of negatively charged amino acids present in all the constituent subunits (Corringer *et al.*, 2000; Imoto *et al.*, 1988). These rings are positioned at both ends of the second membrane-spanning region, and also at an intermediate position which is within the ion channel pore but below the hydrophobic alpha helix of the transmembrane region (Imoto *et al.*, 1988). Figure 1.3.3 shows the positions of the rings of negatively charged residues in the homomeric  $\alpha 7$  nAChR. It has also been demonstrated that these few amino acids are not only necessary but also sufficient to confer cation specificity to an ion channel; mutations at just these three charged amino acids in each subunit can convert a cation channel to an anion channel (Galzi *et al.*, 1992). However, the channel conductance and relative calcium permeability of an nAChR are dependant on the properties of additional amino acids in the channel pore region (Fucile, 2004; Villarroel & Sakmann, 1992).

**Figure 1.4: The ion channel of the nAChR**

Diagram taken from Corringer *et al.*, 2000. This diagram shows the amino acids from the M2 domain of the homomeric  $\alpha 7$  nAChR involved in formation of the ion channel pore. The external ring, intermediate ring and internal ring are composed of negatively charged residues present on all five subunits which confer cation specificity to the channel. Only M2 regions from 2 subunits have been shown for clarity; the other transmembrane regions have also been omitted, except to indicate where the extracellular end of M1 interacts with M2.



## **1.4 Nematode nAChRs**

### **1.4.1 Pharmacology of nematode neuromuscular nAChRs**

Much of the research investigating nematode nAChR pharmacology has been carried out using muscle tissue from *Ascaris suum*; the large size of the worm allows muscle strips and individual muscle cells to be removed with relative ease. The earliest studies were based primarily on muscle contraction assays in response to application of agonist and antagonist drugs (Baldwin & Moyle, 1949). *Ascaris* muscle was shown to contract in response to both acetylcholine and nicotine, and inhibition of ACh-induced contractions could be accomplished by application of tubocurarine and mecamylamine; this provided direct evidence for a nAChR involved in *Ascaris* muscle contractions (Baldwin & Moyle, 1949; Natoff, 1969; Rozhkova *et al.*, 1980).

Electrophysiological techniques have also been widely used to study the pharmacology of native *Ascaris* nAChRs; these recordings utilize extrasynaptic receptors present on the muscle bag cell (Colquhoun *et al.*, 1991; Martin, 1993). Such studies confirmed the primarily nicotinic nature of the ACh response: ACh and nicotine produced dose-dependant depolarisation of the muscle cell, while muscarinic agonists showed little effect (Colquhoun *et al.*, 1991). Mecamylamine was again shown to be a potent antagonist at <1µM concentration, whereas atropine was 100-fold less effective at reducing the ACh response (Colquhoun *et al.*, 1991). Attempts to further characterise the *Ascaris* nAChRs using antagonists selective for different vertebrate nAChR subtypes proved difficult; for example curare and pancuronium had similar efficacy as antagonists of *Ascaris* nAChRs, though these compounds have very different potencies as antagonists of vertebrate neuromuscular nAChRs (Buckett, 1968; Colquhoun *et al.*, 1991).

The use of cholinergic anthelmintic compounds such as morantel, oxantel, pyrantel and levamisole have been perhaps more useful in elucidating the pharmacology of nematode nAChRs. These compounds are potent agonists of nematode neuromuscular nAChRs; depolarisation of muscle cells causing



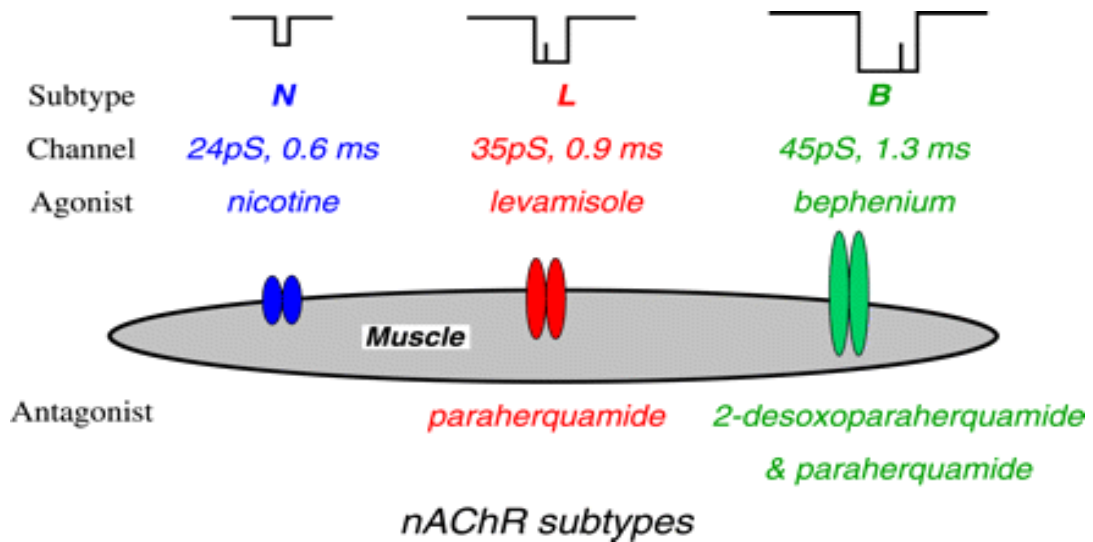
muscle contraction and spastic paralysis underlies their efficacy as anthelmintic agents (Aceves *et al.*, 1970). In addition to this, their selectivity for nematode rather than vertebrate nAChRs produces minimal toxicity when used as therapeutic drugs for the treatment of livestock and humans (Aubry *et al.*, 1970; Eyre, 1970). Electrophysiological recordings of *Ascaris* muscle cell responses to the cholinergic anthelmintics were first performed by Harrow and Gration (1985). This demonstrated dose-dependant increases in cation conductance in response to levamisole, with the EC<sub>50</sub> for levamisole being 5-10 fold lower than for ACh (dependant on method of drug application). Pyrantel and morantel were shown to be even more potent agonists at low concentrations (10nM-10µM) but gave a bell-shaped dose response curve, indicating either desensitization or open channel block at high concentrations (Harrow & Gration, 1985).

Patch-clamp techniques and single-channel recordings have confirmed the mode of action of the cholinergic anthelmintics, and allowed different nAChR subtypes present on *Ascaris* muscle to be characterised; 3 nAChR subtypes with different conductances and mean open times have been described (Qian *et al.*, 2006; Robertson & Martin, 1993; Robertson *et al.*, 1994). These can be distinguished pharmacologically by their relative sensitivities to selective agonists and antagonists, and their properties are summarised in Figure 1.4. The large conductance channel is the B-subtype, most sensitive to buphenium, and can be selectively antagonised using 2-deoxy-paraherquamide. The small conductance channel is the N-subtype, sensitive to agonists nicotine, methyridine and oxantel, and relatively insensitive to antagonist paraherquamide. The channel of medium conductance is the L-subtype receptor, activated by the cholinergic anthelmintics levamisole and pyrantel, and sensitive to the antagonist paraherquamide (Levandoski *et al.*, 2005; Martin *et al.*, 2003; Martin *et al.*, 2004; Qian *et al.*, 2006; Robertson *et al.*, 2002). It has been reported that levamisole and pyrantel resistance in the pig parasite *Oesophagostomum dentatum* may result from loss of the relevant receptor subtype (Robertson *et al.*, 1999; Robertson *et al.*, 2000). The finding that oxantel is selective for a different nAChR subtype than levamisole and pyrantel has important implications for the therapeutic use of these compounds; it is likely that nematodes resistant to one type of cholinergic anthelmintic may still

respond to another, provided it targets a pharmacologically distinct type of nAChR (Martin *et al.*, 2004).

**Figure 1.5: The nAChR subtypes present on *Ascaris* muscle cells**

This diagram is taken from Qian *et al.*, 2006. Single channel recordings from the muscle cells of *Ascaris suum* have demonstrated 3 distinct populations of nAChRs. These channels have different conductances and mean open times, and different pharmacological profiles.



### **1.4.2 Molecular biology of nematode nAChRs**

Although the parasite *Ascaris suum* has been the main focus of studies on nematode nAChR pharmacology, the majority of work on the molecular biology of nematode nAChRs has been confined to *C. elegans*. The main reasons for this are the highly complete and well annotated nature of the *C. elegans* genome sequence, and the ease with which mutants can be made and maintained in the laboratory.

Interest in *C. elegans* nAChR genes began when it was discovered that certain levamisole-resistant *C. elegans* mutants had mutations in genes encoding nAChR subunits (Lewis *et al.*, 1980). Since then, the nAChR gene family has been investigated in depth, and it has been found that *C. elegans* has the largest known nAChR gene family with ~30 subunits (Brown *et al.*, 2006; Jones & Sattelle, 2004). A tree of sequence similarity for the *C. elegans* nAChR gene family is shown in Figure 1.4.2. The nAChR genes of *C. elegans* can be divided into several sub-groups, based on sequence similarity. The *acr-16* group is named after the best characterised member of this group, *acr-16*, which encodes a homomeric, levamisole-insensitive receptor present at the *C. elegans* neuromuscular junction (Mongan *et al.*, 2002; Raymond *et al.*, 2000; Touroutine *et al.*, 2005). The *deg-3* group is nematode-specific and believed to encode mainly neuronal receptors; this group is of interest as it contains *acr-23*, the product of which is targeted by the new AAD anthelmintic compounds (Kaminsky *et al.*, 2008). The *acr-8* group is less well characterised, but contains *lev-8* (also called *acr-13*), a nAChR subunit gene which is known to confer reduced sensitivity to levamisole when mutated (Towers *et al.*, 2005).

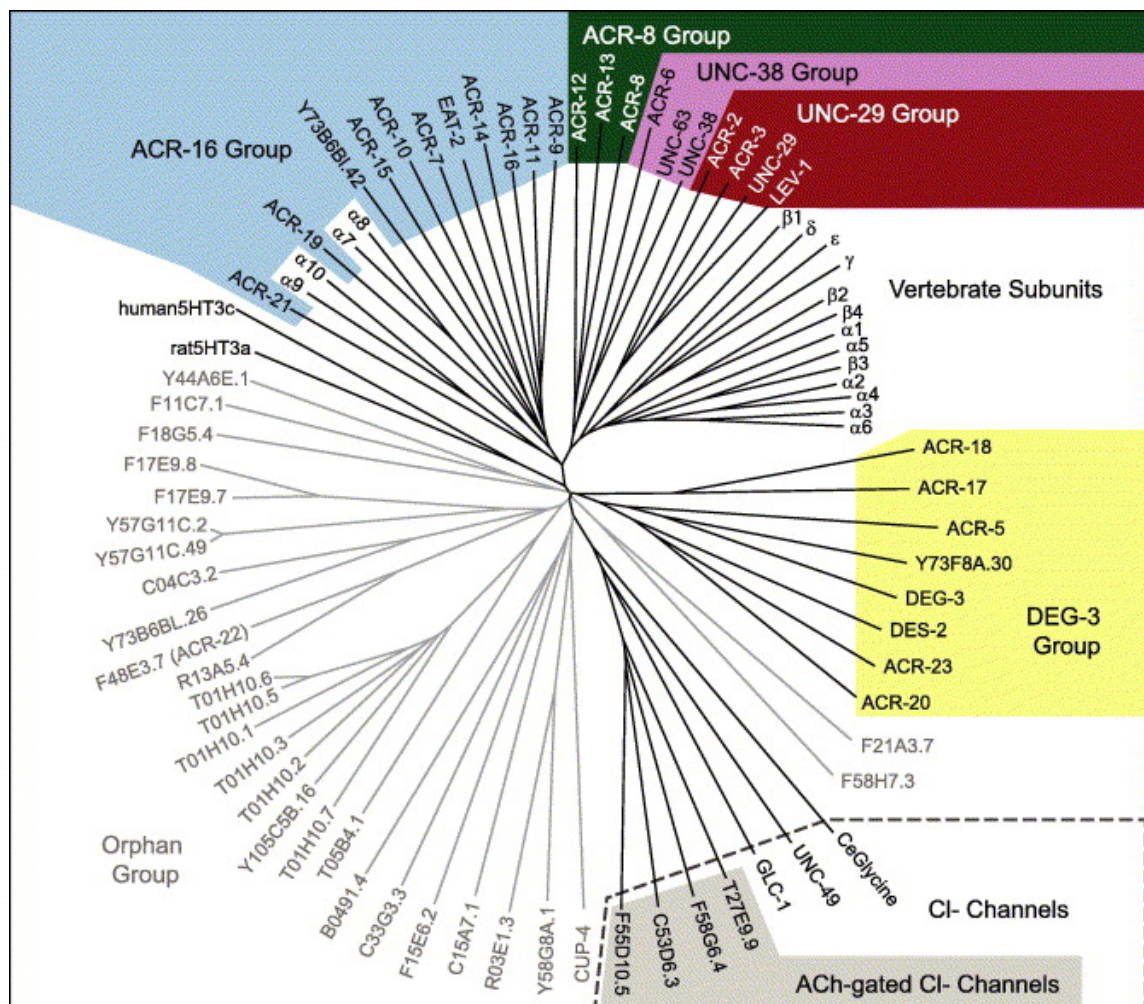
The groups of most relevance to the work presented here are the *unc-38* group and the *unc-29* group. The *unc-38* group contains *unc-38* and *unc-63*, which encode alpha subunits of the levamisole-sensitive nAChR at the *C. elegans* neuromuscular junction (Culetto *et al.*, 2004; Fleming *et al.*, 1997). The *unc-29* group contains *unc-29* and *lev-1*, which encode non-alpha subunits present in the same receptor type (Ballivet *et al.*, 1996; Fleming *et al.*, 1997). Mutations in these subunits all reduce levamisole sensitivity, and heterologous expression of

*unc-38*, *unc-29* and *lev-1* produces a levamisole-sensitive nAChR (Fleming *et al.*, 1997).

Prior to the start of the work presented in this thesis, it had been shown that several parasitic nematodes had mRNA transcripts encoding putative homologues of *unc-38* (Ajuh & Egwang, 1994; Hoekstra *et al.*, 1997; Wiley *et al.*, 1996). A review of potential nAChR sequences present in parasitic nematode EST libraries has yielded further putative homologues of *C. elegans* nAChR subunit genes; however, the localisation and functional significance of such gene products remains almost entirely unknown (Brown *et al.*, 2006). A more recent study which identified and amplified several nAChR genes from the canine hookworm *Ancylostoma caninum* has provided the first evidence of changes in nAChR subunit expression in an anthelmintic resistant population of parasites. McCarthy and colleagues have recently demonstrated significantly lower levels of mRNA encoding the nAChR subunits *unc-38*, *unc-29* and *unc-63* in hookworms which were highly resistant to pyrantel (Kopp *et al.*, 2008). This provided the first direct evidence for the role of these nAChR subunits in determining cholinergic anthelmintic sensitivity in a parasitic species.

**Figure 1.6: The nAChR gene family of *C. elegans***

This diagram is taken from Brown et al. 2006. The cys-loop ligand-gated ion-channel gene family of *C. elegans* contains ~100 genes, of which ~30 encode putative nAChRs. The nAChRs are here shown grouped by sequence similarity (coloured boxes), and have been aligned with the vertebrate nAChR sequences. Also shown are other cys-loop LGIC sequences from *C. elegans*, many of which are "orphans" for which no ligand has yet been identified.



## **1.5 Aims and Objectives**

The pharmacology of the native nAChRs present in muscle membrane from *Ascaris suum* has been well described, but most work on the molecular biology of nematode nAChRs has been performed using the model nematode *C. elegans* (Jones & Sattelle, 2004; Qian *et al.*, 2006). The few studies which have investigated the molecular biology of nAChRs in parasites have utilised species in the same phylogenetic clade as *C. elegans* (Kopp *et al.*, 2008).

The aim of this project was to identify the nAChR subunits from *Ascaris suum* which formed the levamisole-sensitive nAChR, in order to understand the described pharmacology of the native nAChR in terms of the underlying molecular biology. The initial stage of the project aimed to identify which nAChR subunits were likely to be present by searching the genome of a related nematode, *Brugia malayi*, then to amplify subunits with a putative role in the levamisole receptor from *Ascaris suum*.

The next objective was to raise antisera for immunofluorescent labelling, in order to ascertain whether the nAChR subunits were present on the *Ascaris suum* muscle membrane. The final aim of the project was to produce recombinant receptors in a heterologous expression system using the *Ascaris* nAChR subunit cDNA, to allow the pharmacology of the nAChRs to be characterised.

## **Chapter 2: Methods and Materials**

## **2.1 Materials**

### **2.1.1 Nematode tissue**

The nematode tissue used in the initial stages of this project was kindly supplied by Professor Richard Martin and Dr. Alan Robertson of Iowa State University Veterinary College. Heads and muscle strips from freshly collected *Ascaris suum* adults were sent to the University of Bath packaged in dry ice. Upon arrival at the laboratory, the nematode tissue was stored at -80°C to prevent RNA degradation.

Live specimens of *Ascaris suum*, and *Ascaris suum* eggs, were kindly supplied by Emma Kidd and Professor Aaron Maule of Queen's University Belfast. These were sent to the University of Bath in Ascaris Ringer Solution (ARS) at ambient temperature. Upon arrival in the laboratory, eggs were aliquoted into 2ml cryo-vial tubes and snap frozen then stored in liquid nitrogen. Live adult *Ascaris suum* were maintained in the laboratory for 2-3 days in a 5 litre plastic beaker containing 2-3 litres of ARS, kept in a 37°C incubator with daily changes of solution.

**Table 2.1: Composition of *Ascaris* ringer solution**

Ascaris Ringer Solution	4 mM NaCl 5.9 mM CaCl <sub>2</sub> 4.9 mM MgCl <sub>2</sub> 5 mM C <sub>4</sub> H <sub>11</sub> NO <sub>3</sub> /Tris 125 mM NaC <sub>2</sub> H <sub>3</sub> O <sub>2</sub> 24.5 mM KCl pH 7.4
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### **2.1.2 *Xenopus laevis* oocytes.**

Defolliculated *Xenopus laevis* oocytes were obtained from Ecocyte Biosciences, Germany.



**Table 2.2: Plasmids**

Plasmid	Description and use	Supplier
pGEM <sup>®</sup> -T Easy	Amp <sup>r</sup> , 3kb Used in initial cloning of gel-purified PCR products	Promega
pT7TS	Amp <sup>r</sup> , modified pGEM <sup>®</sup> -4Z (Promega) containing UTR regions of <i>Xenopus</i> $\beta$ -globin to enhance expression in <i>Xenopus</i> oocytes	Addgene (original vector from Paul Krieg)

**Table 2.3: Enzymes**

Enzyme	Reaction Buffer (10x)	Supplier
AffinityScript <sup>™</sup> reverse transcriptase	AffinityScript <sup>™</sup> RT buffer: 50mM Tris-HCl (pH 8.3) 750mM KCl 30mM MgCl <sub>2</sub>	Stratagene
Mango Taq DNA polymerase	NH <sub>4</sub> based buffer containing 2mM MgCl <sub>2</sub> and inert dyes	Bioline
Taq DNA polymerase	PCR reaction buffer containing 1.5mM MgCl <sub>2</sub>	Roche
Expand High Fidelity PCR system	Expand High Fidelity reaction buffer containing 1.5mM MgCl <sub>2</sub>	Roche

T4 DNA ligase	Ligase buffer: 30mM Tris-HCl (pH 7.8) 10mM MgCl <sub>2</sub> 10mM DTT 1mM ATP	Promega
T4 DNA ligase	T4 DNA Ligase buffer: 50mM Tris-HCl 10mM MgCl <sub>2</sub> 10mM DTT 1mM ATP pH 7.5	New England Biolabs
BglII	NEB Buffer 3: 100mM NaCl 50mM Tris-HCl 10mM MgCl <sub>2</sub> 1mM DTT pH 7.9	New England Biolabs
EcoRI	NEB Buffer U: 100mM Tris-HCl 50mM NaCl 10mM MgCl <sub>2</sub> 0.025 % Triton X-100 pH 7.5	New England Biolabs
SacI	NEB Buffer 1: 10mM Bis Tris Propane-HCl 10mM MgCl <sub>2</sub> 1mM dithiotheitol pH 7.0	New England Biolabs
SpeI	NEB Buffer 2 (as above)	New England Biolabs

### **2.1.5 Chemicals and reagents**

Unless otherwise specified, all chemicals and general laboratory reagents were of molecular biology grade, and were purchased from Sigma-Aldrich, UK.

## **2.2 Bioinformatics**

### **2.2.1 Reciprocal BLAST searches**

Sequences for the protein-coding regions of all the described *C. elegans* nAChR subunit genes (Jones & Sattelle, 2004) were obtained from the NCBI database (<http://www.ncbi.nlm.nih.gov/>). These sequences were then used to perform searches of the genome sequence of *Brugia malayi* (<http://www.tigr.org/tdb/e2k1/bma1/>) using the TBLASTN algorithm. This algorithm compares translated sequences at the amino acid level rather than making comparisons at the nucleotide level, so is more likely to identify homologous sequences from species which might have different codon usage. The closest match to the *C. elegans* query sequence found in the *Brugia malayi* genome was then used to perform a reciprocal TBLASTN search of the NCBI sequence database <http://www.ncbi.nlm.nih.gov/BLAST>). If this search identified the *Brugia malayi* sequence as most similar to the *C. elegans* nAChR subunit sequence used as the original query, the sequence was identified as genuinely homologous to this *C. elegans* nAChR subunit. This search strategy was then repeated to identify the nAChR subunit genes of another parasitic nematode, *Trichinella spiralis*. The *Trichinella spiralis* genome has been sequenced by the Washington University Genome Sequencing Centre (<http://genome.wustl.edu/tools/blast/>).

### **2.2.2 Sequence alignments and tree construction**

When all putative nAChR sequences had been obtained from the parasite genomes, they were named according to their putative identity and listed along with their *C. elegans* homologues in a WordPad text document. This format is necessary to upload the sequences to the alignment program. The sequences were uploaded to ClustalX (version 1.83) and a complete alignment was

performed; the ClustalX program was also used to produce a boot-strapped neighbour-joined tree from the alignment. The tree of sequence similarity was viewed using TreeView (version 1.6.6).

## **2.3 PCR and Cloning Methods**

### **2.3.1 Primer Design**

The NCBI database (<http://www.ncbi.nih.gov/>) was searched for nicotinic acetylcholine receptor subunit sequences from *Ascaris spp.* One partial sequence was present in the database, with accession number AJ011382; this corresponds to an alpha subunit and putative *unc-38* homologue. The *Ascaris suum* EST database ([http://zeldia.cap.ed.ac.uk/ncbi\\_blast.html](http://zeldia.cap.ed.ac.uk/ncbi_blast.html)) was searched for sequences showing similarity to *C. elegans* nicotinic acetylcholine receptor subunits. Three such sequences were found, but upon translation and sequence comparison in all reading frames, two of these (ASC25431 and ASC07050\_1) were found to contain non-coding regions with many stop codons, so were considered unlikely to encode nAChR subunits. One of the sequences from the EST database, ASC19452\_1, was successfully translated and appeared to encode a putative nAChR subunit. Specific primers were therefore designed to amplify the AJ011382 and ASC19452\_1 sequences. Further degenerate primers were designed to amplify additional nAChR subunits using the UNC-29 and UNC-63 nAChR sequences from *C. elegans* and *Brugia malayi*, based on conserved amino acids. This process is described more fully in section 3.3.

### **2.3.2 RNA extraction**

All equipment used for RNA work was rendered RNase free before use. Pipettes, glassware and surfaces were treated with RNase away reagent (Fluka); plastic tubes, pipette tips and distilled water were treated with diethyl pyrocarbonate then autoclaved.

RNA was extracted from the nematode tissue using Trizol<sup>®</sup> reagent (Invitrogen). The tissue was ground up using a cooled pestle and mortar and 1-4ml Trizol<sup>®</sup> was added (volume depending on sample size). The Trizol<sup>®</sup> and nematode tissue were mixed further in a homogeniser, then decanted into 1.5ml

microcentrifuge tubes. The homogenate was centrifuged at 12000 x g at 4°C for 10 minutes to remove insoluble material, particularly protein from muscle strip samples. The supernatant was transferred to clean eppendorf tubes, then 0.2ml chloroform was added per 1ml of Trizol<sup>®</sup> used in the initial extraction. The tubes were shaken and incubated at ~20°C for 2 minutes, then centrifuged at 12000 x g at 4°C for 10 minutes to separate the lower organic phase containing DNA and protein from the upper aqueous phase containing the RNA. The aqueous phase was transferred to a clean tube and mixed with isopropanol (0.5ml per 1ml Trizol<sup>®</sup> used initially) to precipitate the RNA. After incubation at ~20°C for 10 minutes, the precipitated RNA was collected into a pellet by centrifugation at 12000 x g and 4°C for 10 minutes. The supernatant was discarded, and the RNA pellet washed by resuspension in ethanol (1ml ethanol per 1ml Trizol<sup>®</sup> used initially). The RNA was again collected by centrifugation, at 7500 x g and 4°C for 5 minutes. The RNA was air dried for ~5 minutes, then dissolved in 10-40µl RNase free water.

The RNA was quantified and the purity checked by measuring the absorbance of a 1/100 dilution at 280nm and 260nm using a spectrophotometer. An A260/A280 ratio of ~1.8 indicated a pure RNA preparation. The amount of RNA was calculated by:

$$A_{260} \times \text{dilution factor} \times 40 = \text{amount of RNA in } \mu\text{g/ml}.$$

### **2.3.3 Reverse Transcription**

The mRNA was reverse transcribed into cDNA using AffinityScript<sup>®</sup> reverse transcriptase (Stratagene). The following components were added to an RNase-free 0.5ml tube:

3µl oligo(dT) primer (100ng/µl)

~200ng total RNA (~1ng mRNA)

RNase-free water to 14.2µl

The reaction was heated to 65°C for 5 minutes then slowly cooled to ~20°C to allow (Bektesh *et al.*, 1988) primer annealing.

The following components were added to the reaction:

2µl AffinityScript<sup>®</sup> 10x buffer

2µl DTT

1µl AffinityScript<sup>®</sup> RT (50U/µl)

0.8µl 100mM dNTPs

The reaction incubated at 42°C for 1 hour. After this time the reaction was stopped by incubation at 70°C for 15 minutes.

#### **2.3.4 PCR**

All initial PCR reactions were carried out using Mango-Taq DNA polymerase (Bioline); this preparation contains loading dyes for electrophoresis in the buffer component.

50µl reactions were assembled as follows:

5µl 10x Mango-Taq reaction buffer

2.5µl MgCl<sub>2</sub> (50mM)

1µl Mango-Taq DNA polymerase (1U/µl)

1µl dNTP mix (100mM)

1µl cDNA template

1µl forward primer (final concentration 10 pmol specific primer, 100 pmol degenerate primer)

1µl reverse primer (final concentration 10 pmol specific primer, 100 pmol degenerate primer)

37.5µl dd.H<sub>2</sub>O

PCR conditions used with specific primers were as follows:

2 minutes at 95°C initial denaturation step

35 cycles of:

1 minute denaturation step at 95°C

1 minute primer annealing step at 2-5°C below primer melting temperature

1-2 minutes extension step at 72°C (~1 minute per kilobase of product)

Then a 5 minute extension step at 72°C, followed by cooling to 16°C, maintained until the programme was stopped and the reactions removed from the thermal cycler.

PCR conditions used with degenerate primers were as follows:

2 minutes at 95°C initial denaturation step

10 cycles of:

1 minute denaturation step at 95°C

1 minute primer annealing step at 3°C above primer melting temperature, dropping -0.5°C with each subsequent cycle

1-2 minutes extension step at 72°C (~1 minute per kilobase of product)

30 cycles of:

1 minute denaturation step at 95°C

1 minute primer annealing step at 2°C below lower primer melting temperature

1-2 minutes extension step at 72°C (~1 minute per kilobase of product)

Then a 5 minute extension step at 72°C, followed by cooling to 16°C, maintained until the programme was stopped and the reactions removed from the thermal cycler. A list of primers used for PCR is given in appendix i.

When problems arose due to low expression levels or non-specific amplification, primer pairs internal to the original pair were designed, to carry out nested PCR and reamplify the products of the original PCR reaction.

### **2.2.5 5' and 3' RACE**

When a specific partial sequence coding for a nAChR subunit had been obtained, 5' and 3' RACE (rapid amplification of cDNA ends) was carried out to obtain full length nAChR subunit sequences. 5' RACE used a forward primer specific to the splice leader sequence SL1 known to precede most nematode coding sequences (Bektesh *et al.*, 1988), and a reverse primer complementary to the nAChR partial sequence. 3' RACE used a reverse primer complementary to a known anchor sequence added to the poly-A 3' end of the mRNA during the reverse transcription, and a forward primer specific to the partial nAChR sequence.

### **2.2.6 Agarose gel electrophoresis**

1% w/v gels were made from agarose dissolved in TBE buffer (see appendix i for buffer compositions). The mixture was microwaved until the agarose had dissolved, then allowed to cool to ~40°C before addition of ethidium bromide to a final concentration of 0.5µg/ml. The gel was poured and allowed to solidify,

placed in an electrophoresis tank containing TBE buffer, then 20µl samples of PCR product and 100bp molecular weight markers (Promega) mixed with 5µl 6x loading dye (Promega) were loaded. A current of 50-100mA was applied, and separation of the DNA by molecular weight tracked by migration of the loading dyes down the gel.

### **2.3.7 Gel extraction**

If PCR products of the correct size were observed on the agarose gel, they were carefully excised with a clean razor blade, and the DNA extracted using a gel extraction kit (Qiagen). The gel slice was dissolved in 3 volumes of buffer to 1 volume of gel by incubation at 50°C for ~10 minutes. 1 volume of isopropanol to 1 volume of gel was added, then the mixture was applied to a DNA binding spin column and centrifuged at 13000 x g for 1 minute. The bound DNA on the spin column was then washed with 750µl ethanol-based buffer and subsequently dried by further centrifugations for 1 minute at 13000 x g. 30µl nuclease-free d.H<sub>2</sub>O was applied to the spin column, and the eluted DNA collected by a final centrifugation step at 13000 x g for 1 minute.

### **2.3.8 Ligation reactions**

The gel extracted DNA was then ligated into pGEM-T Easy vector (Promega).

Ligation reactions were assembled as follows:

5µl 2x Rapid Ligation Buffer

1µl pGEM-T Easy vector (50ng)

3µl gel-extracted DNA

1µl T4 DNA ligase (3U/µl)

The reactions were incubated for either 1 hour at room temperature or overnight at 4°C.

### **2.3.9 Preparation of competent cells**

A scraping of cells from a permanent culture of stock XL1 blue strain *E.coli* was inoculated into 100ml of LB broth in a 1 litre conical flask and incubated at



37°C with moderate agitation for ~3 hours until the OD<sub>600</sub> reached ~0.3 (indicating that the number of viable cells < 10<sup>8</sup>/ml).

The culture was transferred to sterile 50ml tubes and cooled on ice for 10 minutes, then centrifuged at 2700 x g at 4°C for 10 minutes. The supernatant was discarded and the tube inverted to remove any remaining media from pellet of cells. The bacterial cells were resuspended in 30 ml of 80mM MgCl<sub>2</sub>-20mM CaCl<sub>2</sub> solution which had previously been cooled on ice, then collected by a further centrifugation at 2700 x g at 4°C for 10 minutes. After again discarding the supernatant and draining by inversion of the tube, the cells were resuspended in 2ml 0.1M CaCl<sub>2</sub> (cooled on ice) per 50ml of original culture. Glycerol was added as a cryoprotectant (15-25% by volume) then the competent cells were dispensed into 200µl aliquots and snap-frozen in liquid N<sub>2</sub> prior to storage at -80°C.

#### **2.3.10 Transformation of competent cells**

An aliquot of competent cells was thawed on ice. 200µl cells were added to the DNA insert/vector product of the ligation reaction, then left on ice for 30 minutes. The cells were heat-shocked at 42°C for 5 minutes, then added to 1ml of LB medium and incubated at 37°C for 1 hour. LB/ampicillin agar plates were spread with 10µl IPTG (20%w/v) and 50µl X-Gal (2%, Promega), which was left to absorb at 37°C for 1 hour. The transformed cells were collected by centrifugation (13000 x g for 1 minute), resuspended in residual media, then spread onto the prepared agar plates. The plates were incubated overnight at 37°C.

Colonies containing recombinant plasmids with the DNA insert of interest were white in colour; these were used to inoculate LB/ampicillin broth and cultured overnight at 37°C.

#### **2.3.11 Plasmid extraction and digest**

Plasmids were extracted from overnight cultures of transformed *E. coli* using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich). 1.5ml of overnight culture was centrifuged at 13000 x g for 1 minute. The cells were resuspended in 200µl resuspension solution, then lysed by addition of 200µl alkaline-SDS lysis

buffer. After 5 minutes, 350µl high salt neutralisation/binding buffer was added, then the cell debris removed by centrifugation at 13000 x g for 10 minutes. The supernatant (containing plasmids) was added to a DNA binding spin column (silica membrane which binds DNA in the presence of high salt concentrations, prepared according to the manufacturer's instructions) and centrifuged for 1 minute at 13000 x g. The DNA bound to the spin column was then washed by addition of 750µl ethanol-based wash solution and centrifugation at 13000 x g for 1 minute; an additional centrifugation step was then performed for the same time and speed to remove all traces of wash solution from the spin column. The spin column was transferred to a clean collection tube, and the DNA eluted in 100µl nuclease-free dd.H<sub>2</sub>O.

To confirm the presence of an insert in the extracted plasmids, a restriction enzyme digest was performed, using the enzyme EcoR1, as the vector used has restriction sites for this enzyme at either side of the insert site. Reactions were assembled as follows:

5µl buffer (x10)

1-5µl plasmid preparation

0.2µl BSA

0.5µl enzyme

dd.H<sub>2</sub>O to 20µl

The reactions were incubated at 37°C for 1-4 hours, then 5µl loading buffer was added and the sample separated by agarose gel electrophoresis. If a DNA insert band corresponding to the size of the initial PCR product could be seen, the plasmid preparation was sent for sequencing, using M13 forward and reverse sequencing primers. A BLAST search was then performed to compare the resulting sequence with those in the NCBI database (<http://www.ncbi.nih.gov/BLAST>), to determine whether the cDNA sequence which had been amplified and cloned coded for a nicotinic acetylcholine receptor subunit.

### **2.3.12 Proof-reading and sequence deposition**

Following successful amplification and cloning of a complete nAChR subunit sequence, the PCR reaction was repeated using new specific primers and the

Expand High Fidelity PCR system (Roche) to ensure the accuracy of the sequence amplification. The PCR product was then cloned as described previously, and plasmid from 3 or 4 separate clones was sequenced to ensure consensus. The sequences were then deposited in the NCBI database, and assigned accession numbers.

### **2.3.13 Sub-cloning into expression vector pT7TS**

The pT7TS plasmid is an expression vector optimised for *Xenopus* oocyte expression by the insertion of the *Xenopus*  $\beta$ -globin 5' and 3' UTR sequence. This plasmid originated in the laboratory of Paul Krieg, University of Texas, and can be obtained from Addgene (plasmid 17091).

The sequences of *Ascaris suum* *unc-38* and *unc-29* were analysed using software from Biology Workbench 3.2 (<http://seqtool.sdsc.edu/CGI/BW.cgi>) to ensure that the restriction sites needed for sub-cloning into pT7TS, BglII and SpeI, were not present in the cDNA sequences. Primers were designed to amplify the full-length nAChR subunit cDNAs and add the appropriate restriction sites to the ends of the sequences; a full list of primers used is given in Appendix i. PCR was performed as described in section 2.2.4, using 1  $\mu$ l of pGEM-T Easy plasmid containing the nAChR subunit sequence as template. The PCR product was then cloned into pGEM-T Easy as described in sections 2.2.10 and 2.2.11.

1-3  $\mu$ g of both the pGEM-T Easy plasmids containing the nAChR cDNAs plus restriction sites, and the pT7TS vector, were digested using the restriction enzymes BglII and SpeI as described on the New England Biolabs double-digest finder website

(<http://www.neb.com/nebecomm/DoubleDigestCalculator.asp>). Both the digested vector and nAChR sequences were purified by agarose gel electrophoresis (section 2.2.6). Both the plasmid and the insert were quantified by obtaining the A260 value using a spectrophotometer, then they were ligated together in a 1:3 vector:insert ratio using New England Biolabs T4 DNA ligase as described in the manufacturer's instructions. Competent cells were transformed using the product of the ligation reaction, then plated out and colonies picked and grown overnight as described in section 2.2.10. Plasmids

were purified (section 2.2.11) and sequenced to ensure that the pT7TS plasmids now contained the *Ascaris suum* *unc-38* and *unc-29* sequences.

#### **2.3.14 Real-time PCR**

Primers were designed to amplify ~200bp regions of the N-terminal regions of the *Ascaris suum* nAChR subunits *unc-38*, *unc-29* and *unc-63*. An additional primer set was designed to amplify a region of similar length from the *Ascaris suum*  $\beta$ -tubulin sequence obtained from the GenBank database (accession number FE918860). All primers had a similar length, GC content and melting temperature, and the primer sequences are listed in Appendix i. RNA was extracted from *Ascaris* heads and body wall sections as described in section 2.3.2. Reverse transcription reactions were set up as described in section 2.3.3, ensuring that all reactions contained the same amount of RNA. The primers and cDNA were first tested by ordinary PCR, using the same reaction conditions which would be used for real-time PCR. Real-time PCR was performed using a Stratagene Mx3000P thermal cycler, and amplification plots and analysis were generated using the accompanying computer software. Each real-time PCR run included negative control reactions in which the cDNA template had been omitted, to control for any potential contamination.

Real-time PCR reactions were set up as follows:

25 $\mu$ l iTaq<sup>TM</sup> SYBR<sup>®</sup> Green supermix with ROX (Bio-Rad)

22 $\mu$ l nuclease free d.H<sub>2</sub>O

1 $\mu$ l forward primer

1 $\mu$ l reverse primer

1 $\mu$ l cDNA template

PCR conditions used with real-time PCR primers:

2 minutes at 95°C initial denaturation step

40 cycles of:

1 minute denaturation step at 95°C

45 second primer annealing step at 58°C

1 minute extension step at 72°

## **2.4 Antibody Methods**

**Table 2.4: Immunohistochemistry buffers and solutions**

PBS	10mM phosphate buffer, 2.7mM potassium chloride and 137mM sodium chloride, pH 7.4
column wash buffer	20mM Na <sub>2</sub> HPO <sub>4</sub>
acid elution solution	50mM glycine HCl (pH 2.3)
neutralisation solution	1M Tris (pH10.8)
column storage buffer	20mM Na <sub>2</sub> HPO <sub>4</sub> , 0.5M NaCl, 0.01% NaN <sub>3</sub>
PBS-tween	PBS with 0.1% v/v Tween-20
coating buffer	15mM Na <sub>2</sub> CO <sub>3</sub> , 35mM NaHCO <sub>3</sub>
blocking solution	1% BSA in PBS-Tween
acetate/citrate buffer	1.66M sodium acetate, 33mM citric acid, pH6, diluted 1:20 in dd.H <sub>2</sub> O
TMB	10mg Tetramethyl Benzidine in 1ml DMSO
stop solution	1.84M H <sub>2</sub> SO <sub>4</sub>
PBS-Triton X	PBS with 0.1% v/v Triton X
fixative solution	4% paraformaldehyde in PBS Triton-X

antibody dilution solution	PBS with 0.5% Triton-X, 0.1% BSA, 0.05% NaN <sub>3</sub>
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#### **2.4.1 Production of antisera**

Subunit-specific antigenic peptides were predicted from the translated subunit sequence, using the DNA Star Protean software. A hydrophilic, antigenic region of ~15 amino acids with a high surface probability was selected from the N-terminal region of each sequence as an appropriate peptide for raising antisera. This region of ligand-gated ion channel sequence was successfully used for the production of antisera by Delany et. al. (1998). The selected peptides were synthesised as MAP antigens by Alta Biosciences, UK, and were dissolved in sterile PBS at a concentration of 1mg/ml before use. The peptide specific for AsUNC-29 was sent to Harlan, UK, for the production of antisera in rabbit. The peptides specific for AsUNC-38 and AsUNC-63 were sent to Sigma-Genosys, USA, for the production of antisera in goats.

#### **2.4.2 Affinity purification of antisera**

Peptide in a controlled pore glass (CPG) column format was supplied by Alta Biosciences, UK, for the purpose of affinity purification. All affinity purification procedures were carried out at 4°C. The composition of the buffers and solutions used are listed in Table 2.4.

The column was washed using approximately 50ml (ie.10x column volume) column wash buffer. Tubing and a pump were connected to the column to create a recirculating system. 5ml of antiserum was loaded into the column and allowed to recirculate overnight at a flow rate of 2-4ml/minute. The serum was then drained from the column, and the column was washed with 50ml wash buffer. The antibody bound to the column was eluted using acid elution solution. 10x 1ml aliquots were collected and immediately neutralised with 200µl neutralisation solution. The column was again washed with 50ml wash solution, and was then stored at 4°C under column storage buffer.

The protein content of each of the 10 aliquots eluted from the column was then crudely measured by A280. The aliquots with the highest protein content were

assumed to contain most of the eluted antibody, and were pooled then dialysed overnight against PBS.

### **2.4.3 ELISA**

A 96-well plate was coated with 100µl of peptide in each well, diluted to 10µg/ml in coating buffer (see Table 2.4) and incubated at 37°C for 1 hour, then at 4°C overnight. The plate was washed 3x 15 minutes with PBS-Tween, with rigorous expulsion of liquid from the wells between washes. The wells were filled with 200µl blocking solution (Table 2.4), and incubated on a shaker for 1 hour. The plate was then washed with PBS-Tween, 3x 15 minute washes.

Primary antisera (antiserum from terminal bleed, affinity purified antibody, and pre-immune serum) was placed in the first row of wells at 1:100 dilution in PBS Tween. This was serially diluted in PBS-Tween across the plate. The plate was incubated on a shaker for 2 hours at cool room temperature, then washed for 3x 15 minutes in PBS-Tween, with rigorous expulsion of liquid from the wells between washes.

Peroxidase-conjugated secondary antiserum (anti-rabbit or anti-goat IgG HRP) was diluted 1:2000 in PBS-Tween, and 100µl added to each well. The plate was incubated on a shaker for 2 hours at cool room temperature, then washed for 3x 15 minutes in PBS-Tween, and for 1x 15 minutes in PBS.

The plate was developed using a solution of 0.5ml TMB and 10µl H<sub>2</sub>O<sub>2</sub> (30% v/v) in 49.5ml of acetate/citrate buffer. 100µl of this solution was added to each well, and the reaction observed until the colour developed. The reaction was then stopped by addition of 50µl stop solution to each well.

A plate reader was used to measure the A450 values for each well. These values were the plotted as a graph of A450 against dilution.

### **2.4.4 Preparation of muscle cells for immunofluorescence**

Muscle cells were loosened from the cuticle using an adaptation of the collagenase treatment protocol described by Johnson and Stretton (1985). The worms were pinned in a dissection tray, injected with 500µl 0.5% w/v crude bacterial collagenase (Sigma) in ARS (100µl at 5 equidistant points along the worm), and incubated at 37°C for 1 hour. Worms were then rinsed and the

cuticle opened under PBS, allowing the muscle cells to be rinsed out and collected using a Pasteur pipette. The muscle cells were allowed to settle slowly out of suspension, and the PBS was gently removed and replaced by fixative solution. The cells were fixed for ~12 hours, then washed 3 times in PBS-Triton X, allowing the cells to settle slowly out of solution as centrifugation can damage cell structure. Sections of cuticle with nerve cords attached were also dissected from the specimen after collagenase digestion, and were flat-fixed for ~24 hours between glass slides then washed 3 times in PBS-Triton X.

#### **2.4.5 Immunofluorescence**

Fixed *Ascaris suum* material was labelled with antibodies for indirect immunofluorescence using an adaptation of the methods described by Brownlee *et al.* (1993). Affinity purified antibody was diluted 1:100 in antibody dilution solution (see Table 2.4), and 5ml was added to muscle cells or cuticle/nerve cord preparations in 15ml tubes. The muscle cells were incubated in primary antibody overnight on a roller at 4°C. The cuticle strips bearing nerve cords were incubated in the same manner for ~24 hours. The specimens were then washed for 3x 15 minutes in PBS Triton-X.

Secondary antibody (either FITC conjugated anti-goat IgG or Alexafluor 594 conjugated anti-rabbit IgG) was diluted 1:200 in antibody dilution solution and added to muscle cells or cuticle/nerve cord preparations in 15ml tubes. After 4-5 hours incubation at 4°C on a roller, the muscle cells were washed 3x15 minutes in PBS Triton-X, and mounted on slides under coverslips. Cuticle strips bearing nerve cords were subjected to a longer incubation in secondary antibody, ~12 hours, before washing and mounting. All prepared and mounted *Ascaris suum* material was viewed using a Zeiss 510 confocal laser scanning microscope.



## **2.5 *Xenopus* oocyte expression and electrophysiology**

### **2.5.1 Linearisation of plasmid DNA**

. The *Ascaris suum* *unc-38* and *unc-29* sequences were analysed using software from the Biology Workbench 3.2 website (<http://seqtool.sdsc.edu/CGI/BW.cgi>) to match restriction sites from the pT7TS plasmid downstream of the *Xenopus* globin 3' UTR to enzymes which would not cut the insert sequence itself. SacI was found to be appropriate for the linearisation of both *unc-38* and *unc-29* containing plasmids.

Restriction digests were set up in nuclease free 1.5ml microcentrifuge tubes:

74µl nuclease free H<sub>2</sub>O

10µl 10x reaction buffer

1µl BSA (10mg/ml)

5µl restriction enzyme

10µl plasmid (~1µg/µl)

The reactions were incubated at 37°C for 2-3 hours.

The linear DNA was then purified by phenol-chloroform extraction. 200µl nuclease-free H<sub>2</sub>O and 300µl phenol:chloroform:isoamyl alcohol (25:24:1, Sigma-Aldrich) were added to the digest reaction mixture, and vortex mixed. Centrifugation at 13000 x g for 2 minutes was carried out to allow phase separation. The upper aqueous layer was transferred to another nuclease-free tube, and an equal volume of chloroform added. The sample was again vortex mixed then centrifuged at 13000g for 2 minutes. The aqueous layer containing the DNA was removed to another nuclease-free tube, and 30µl 3M sodium acetate and 720µl of absolute ethanol were added. The tube was placed at -20°C overnight to allow the DNA to precipitate. The DNA was recovered by centrifugation at 13000 x g and 4°C for 15 minutes. The supernatant was removed, and the DNA allowed to dry for ~5 minutes before resuspension in 10µl nuclease-free H<sub>2</sub>O.

### **2.5.2 *In vitro* transcription**

*In vitro* transcription of the linear DNA was carried out using the T7 mMESSAGE mMACHINE kit (Ambion). This kit transcribes the sequence starting from the T7 site, which in pT7TS is upstream of the insert and the

*Xenopus*  $\beta$ -globin UTRs. Transcription reactions were set up in RNase-free microcentrifuge tubes:

3 $\mu$ l nuclease-free H<sub>2</sub>O

10 $\mu$ l 2x NTP/Cap

2 $\mu$ l 10x Reaction Buffer

3 $\mu$ l linear template DNA

2 $\mu$ l enzyme mix

The reactions were incubated at 37°C for 2 hours, then the DNA template removed by addition of 1 $\mu$ l DNaseI and a further incubation at 37°C for 15 minutes. The reaction was then stopped by addition of 15 $\mu$ l ammonium acetate stop solution, and the volume was made up to 150 $\mu$ l by addition of nuclease-free H<sub>2</sub>O.

The cRNA was then purified by phenol-chloroform extraction. An equal volume of phenol:chloroform (5:1, Sigma-Aldrich) was added to the reaction mixture, vortex mixed, then centrifuged at 13000 x g for 2 minutes to allow phase separation. The upper aqueous phase was removed to a clean nuclease-free microcentrifuge tube, and an equal volume of chloroform added. The sample was again vortex mixed then centrifuged at 13000 x g for 2 minutes. The aqueous layer containing the RNA was removed to another nuclease-free tube, and 150 $\mu$ l isopropanol added. The RNA was precipitated by placing at -20°C overnight, then recovered by centrifugation at 13000 x g and 4°C for 15 minutes. The supernatant was removed and the RNA dried at room temperature for ~5 minutes before resuspension in 10 $\mu$ l nuclease-free H<sub>2</sub>O. The concentration of RNA was quantified by A260 using a spectrophotometer. The concentration of each subunit cRNA was adjusted to 1 $\mu$ g/ $\mu$ l by dilution with nuclease free water.

### **2.5.5 Microinjection of oocytes**

The oocytes were incubated in incubation medium (table 2.5). All manipulations of oocytes were carried out using 3ml plastic pasteur pipettes and a fine paintbrush. The oocytes were stabilised during injection procedures by placing them in 35mm petri dishes onto which grooves of the correct width to

accommodate oocytes had been scored using a heated razor blade. Injection needles were pulled from 3.5nl glass capillary tubes (World Precision Instruments, USA) using a p-2000 needle puller (Sutter Instrument Company, USA) with the settings heat=335, fil.=4, vel.=45, del.=250, pull=150. The needle tips were broken off using fine forceps, and the needles back-filled with mineral oil and attached to the Drummond 'Nanoject' microinjector. The needle was filled with the appropriate cRNA solution, and each oocyte was injected with 50nl of cRNA (approximately 40ng). The oocytes were placed in 35mm petri dishes in fresh incubation medium and incubated at 18 °C for 48-72 hours, with daily changes of medium. After 72 hours, oocytes were maintained at 4 °C up to a maximum time of 5 days post-injection.

#### **2.5.6 Two-electrode voltage clamp**

Recordings were made 2-4 days after RNA injection. Microelectrodes were pulled from 1.17mm interior diameter borosilicate capillary tubing (Warner instruments, order no. 64-0805) using a p-2000 needle puller (Sutter Instrument Company, USA) with the settings heat=355, fil.=4, vel.=50, del.=225, pull=75. The microelectrodes were backfilled with 3M KCl solution using a long fine needle. After attachment to the headstage, the resistance of the electrodes was checked to ensure it was between 0.5 and 5 $\Omega$ , and the voltage of each electrode was zeroed.

The eggs were placed in the bath, impaled with the electrodes, and continuously perfused with SOS to which 0.5 $\mu$ M atropine had been added, to inhibit any endogenous muscarinic receptors which may also respond to acetylcholine. Current was applied sufficient to hold the membrane potential at -60mV, applied and measured using a GeneClamp 500 amplifier (Axon Instruments). Perfusion was delivered by a ValveLink 8.2 digitally controlled perfusion system (AutoMate Scientific, USA). Agonists and antagonists (obtained from Sigma) were dissolved to the required concentrations in SOS. Agonists were perfused for 5 second intervals, then washed off by perfusion of SOS. Antagonists were perfused for 30 seconds prior to application of agonist; wash off was achieved by further perfusion of SOS. Agonist doses were applied at 3

minute intervals to allow receptor recovery from any desensitisation. Responses to cholinergic agonist application were measured as changes in injected current needed to maintain membrane holding potential. The data were digitized using the Digidata 1322A (Axon Instruments); responses were recorded and quantified using the pClamp software Clampex 8.2 and Clampfit 8.2. Data were then exported to GraphPad Prism for statistical analysis. Data were normalised to the response to 100 $\mu$ M ACh for each individual oocyte, and a sigmoidal dose-response curve for each agonist was fitted using non-linear regression.

**Table 2.5: Solutions used for oocyte incubation and electrophysiology**

Standard Oocyte Saline (SOS)	100mM NaCl 1mM MgCl <sub>2</sub> 5mM HEPES 2mM KCl 1.8mM CaCl <sub>2</sub> pH 7.5
Incubation medium	Standard oocyte saline 2.5mM pyruvate 100 $\mu$ g/ml penicillin 100 $\mu$ g/ml streptomycin 50 $\mu$ g/ml gentomycin

## **Chapter 3: Bioinformatics**

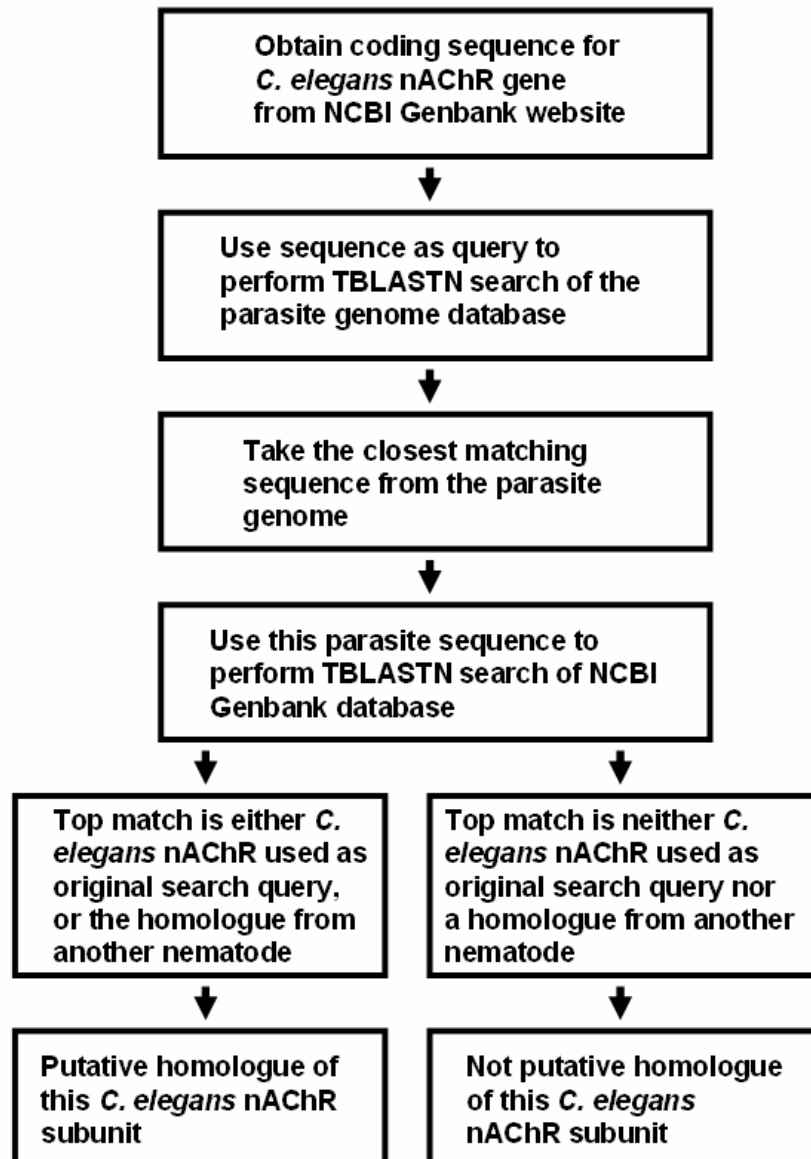
### **3.1 Introduction**

The model nematode *C. elegans* has the largest known nAChR gene family, with ~30 nAChR subunit genes (Brown *et al.*, 2006). However, prior to the start of the work presented here, little was known about the nAChR gene families of other nematodes, though putative homologues of *unc-38*, which encodes an  $\alpha$ -subunit of *C. elegans* levamisole-sensitive nAChR, had been identified in a few nematode parasites (Ajuh & Egwang, 1994; Hoekstra *et al.*, 1997; Walker *et al.*, 2001). In insects, the nAChR gene family found in the model dipteran *Drosophila melanogaster* has provided a basis from which the genomes of other insects can be searched to identify nAChR subunit genes based on their homology to *Drosophila* sequences. This approach has been successfully applied to insect groups phylogenetically distinct from flies, such as the honey bee *Apis mellifera*, the flour beetle *Tribolium castaneum* (Jones & Sattelle, 2006; Jones & Sattelle, 2007).

As the genome sequence of *Ascaris suum* was unavailable at the beginning of this project, it was decided that a search of the genome of a related parasite, *Brugia malayi*, may provide useful information about which nAChR subunit genes were likely to be present in *Ascaris suum*. *Brugia malayi* and *Ascaris suum* both belong to the clade III phylogenetic group, whereas *C. elegans* belongs to the phylogenetically distinct clade V (Blaxter *et al.*, 1998). As the nAChR gene family of the model nematode *C. elegans* has been extremely well described, *C. elegans* nAChR subunit sequences were used as query sequences for performing searches of the parasite genome (Jones *et al.*, 2007; Jones & Sattelle, 2004). When this search had been completed, the search for nAChR subunit genes was extended to the genome of another phylogenetically distinct parasite, the clade I nematode *Trichinella spiralis*. A summary of the steps involved in performing a reciprocal BLAST search to identify putative nAChR subunit genes is shown in Figure 3.1. The methods are described more fully in section 2.2.

**Figure 3.1: Identification of putative homologues of *C. elegans* nAChR genes in parasite genomes**

The flowchart summarises the methods used to identify putative homologues of *C. elegans* nAChR subunit genes from the genome sequences of *Brugia malayi* and *Trichinella spiralis*.



### **3.2 The nAChR gene families of *Brugia malayi* and *Trichinella spiralis***

Although *C. elegans* has the largest known nAChR gene family, with ~ 30 genes, searches of the genomes of *Brugia malayi* and *Trichinella spiralis* reveal far fewer apparent nAChR subunits: 9 and 8 respectively. These results are summarised in Table 3.1, and are presented as a tree of sequence similarity in Figure 3.2. The tree of sequence similarity is based on a ClustalX alignment of the translated parasite nAChR subunit sequences against their putative homologues from *C. elegans*.

Notable absences of particular relevance to the work presented here are certain proposed components of the levamisole receptor; *lev-1* and *lev-8* were not found in the genomes of either parasite species (Fleming *et al.*, 1997; Lewis *et al.*, 1980; Towers *et al.*, 2005). However, the other nAChR subunits thought to comprise the *C. elegans* levamisole-sensitive receptor, *unc-38*, *unc-29* and *unc-63*, are present and highly conserved in both parasites (Culetto *et al.*, 2004; Fleming *et al.*, 1997). Another notable absence in terms of anthelmintic targets was *acr-23*. The nAChR subunit this gene encodes is believed to be the target of the new cholinergic anthelmintic compounds the AADs, but *acr-23* was not present in the genomes of these clade III and clade I parasites (Kaminsky *et al.*, 2008). A further difference between the nAChR gene families of the two parasites investigated and that of *C. elegans* is the apparent absence of *acr-16*, which encodes a subunit forming a homomeric levamisole-insensitive neuromuscular nAChR (Touroutine *et al.*, 2005). The *acr-16* group of *C. elegans* contains 11 subunits, whereas *Brugia malayi* and *Trichinella spiralis* only possess one subunit sequence each from this group; it is a different one in each species, and neither appears truly homologous to *acr-16*. All the other nAChR subunit groups from *C. elegans*, which have been described on the basis of sequence similarity, also contain fewer members in the parasites. An additional nAChR subunit not present in *C. elegans* is found in the *Brugia malayi* genome, and is also the only nAChR subunit sequence present in the *Ascaris* EST library. This divergent subunit is most similar to nAChR subunit G



from the pond snail *Lymnaea stagnalis* (van Nierop *et al.*, 2006), and is apparently conserved amongst clade III nematodes, though it was not found in the genome of the clade I nematode *Trichinella spiralis*. With the agreement of the annotators of the *C. elegans* nAChR subunits (Andrew Jones and Jonathon Hodgkin, personal communication) this sequence was named *acr-26*; *acr-* is the prefix given the nAChR subunits not already named by phenotype, and 26 is the logical designation for a nAChR subunit additional to the 25 *acr*'s already named in *C. elegans* (Jones *et al.*, 2007).

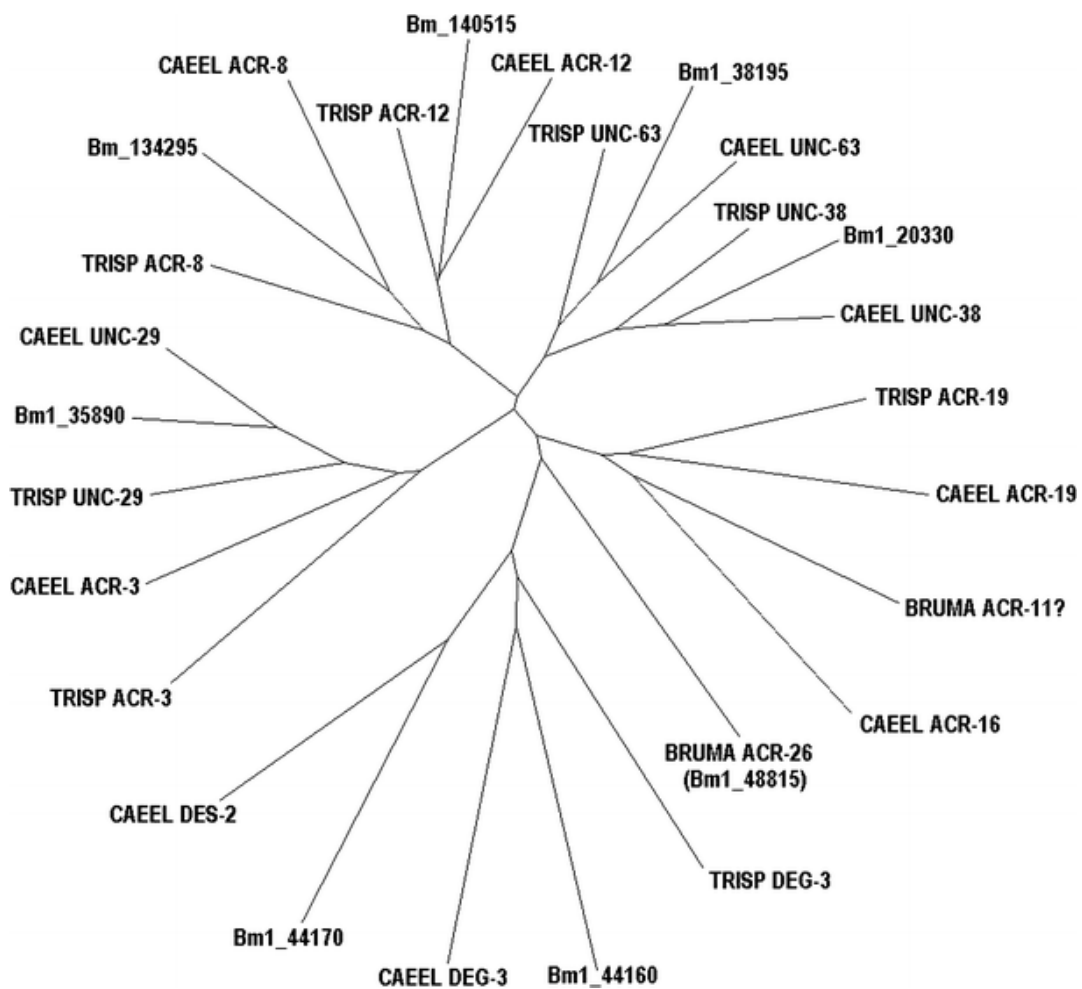
**Table 3.1: Summary of nAChR subunit sequences from in *Brugia malayi* and *Trichinella spiralis*: a comparison with *C. elegans***

The complete list of *C. elegans* nAChR subunits used for this comparison, the nomenclature used, and the assignment of nAChR subunits to groups based on sequence similarity, is as described by Jones *et al.* (2007).

<i>C. elegans</i> nAChR subunit group	Present in <i>B. malayi</i>	Absent from <i>B. malayi</i>	Present in <i>Trichinella spiralis</i>	Absent from <i>T. spiralis</i>
<i>unc-38</i> group	<i>unc-38</i> <i>unc-63</i>	<i>acr-6</i>	<i>unc-38</i> <i>unc-63</i>	<i>acr-6</i>
<i>unc-29</i> group	<i>unc-29</i>	<i>lev-1</i> <i>acr-2</i> <i>acr-3</i>	<i>unc-29</i> <i>acr-3</i>	<i>lev-1</i> <i>acr-2</i>
<i>acr-8</i> group	<i>acr-8</i> <i>acr-12</i>	<i>lev-8</i>	<i>acr-8</i> <i>acr-12</i>	<i>lev-8</i>
<i>deg-3</i> group	<i>deg-3</i> <i>des-2</i>	<i>acr-5</i> <i>acr-17</i> <i>acr-18</i> <i>acr-20</i> <i>acr-23</i>	<i>deg-3</i>	<i>des-2</i> <i>acr-5</i> <i>acr-17</i> <i>acr-18</i> <i>acr-20</i> <i>acr-23</i>
<i>acr-16</i> group	<i>acr-11?</i>	<i>eat-2</i> <i>acr-7</i> <i>acr-9</i> <i>acr-10</i> <i>acr-14</i> <i>acr-15</i> <i>acr-16</i> <i>acr-19</i> <i>acr-21</i> <i>acr-25</i>	<i>acr-19</i>	<i>eat-2</i> <i>acr-7</i> <i>acr-9</i> <i>acr-10</i> <i>acr-11</i> <i>acr-14</i> <i>acr-15</i> <i>acr-16</i> <i>acr-21</i> <i>acr-24</i>

**Figure 3.2: The nAChR gene families of *Brugia malayi* and *Trichinella spiralis***

Boot-strapped neighbour-joining tree generated from a ClustalX alignment of the translated nAChR sequences found in the genome sequences of *B. malayi* and *T. spiralis*, aligned with their *C. elegans* homologues. Genes found by Ghedin et al.2007 are described by the published locus number; otherwise our own proposed nomenclature is used, utilising the *C. elegans* gene names along with a prefix to designate the nematode species (Williamson *et al.*, 2007).



### **3.3 The use of consensus nAChR sequences for primer design**

The primary reason for carrying out searches for nAChR subunit sequences in the genome of *Brugia malayi* was to predict which nAChR subunits were likely to be present in *Ascaris suum*, so attempts could be made to amplify these using PCR. Amplification of nAChR subunits for which partial sequences were not already present in the databases would require the use of degenerate primers, which are designed on regions of amino acid sequence conserved between species. Alignments were made of the translated sequences of well-conserved nAChR subunits from *C. elegans* and *Brugia malayi* using ClustalW. Figure 3.3 shows ClustalW alignments used for the design of degenerate primers designed to amplify *unc-29* and *unc-63*. Conserved regions featuring amino acids with the fewest possible combinations of codons were chosen for primer design, to minimise the degeneracy.

**Figure 3.3: ClustalW alignments of the nAChR subunit sequences UNC-29 and UNC-63 from *C. elegans* and *B. malayi*.**

The amino acid sequences of UNC-29 and UNC-63 are well-conserved between *C. elegans* (lower rows, labelled with subunit name) and *B. malayi* (upper rows, labelled "brugia"). Conserved residues are indicated by an asterisk. Regions used for degenerate primer design are highlighted, with arrows above the sequence indicating the direction of amplification for which the primer would be used.

a) UNC-29

```

brugia      -----SDEEERLMVDVFRGYNSLIQPVRNLNETPIIVKI 34
unc29      MRTNRLSWILVLSVVI FLVIINTINASDDEERLMVDVFRGYNSLIQPVRNSELPLIVKM 60
              ** :***** . * *:***:

brugia      >
ALQLALLINVDEKDKIMHTNVWLTL-WHDFQMRWNPVDYGEIQNIRVAPDKVWLPDIVLF 93
unc29      ALQLVLLINVDEKDKVMHTNVWLTLQWHDFQMKWNPVNYGEIKQIRVSPDKVWLPDIVLF 120
              **** .*****:***** *****:****:****:****:*****

brugia      < >
NAADGNYEVSFMCNVVINYKGEMLWVPPAIYKSSCIIDVEFFPFDEQCHLIFGSWITYNE 153
unc29      NNADGNYEVSFMCNVVINHKGDMLWVPPAIYKSSCIIDVEFFPFDEQVCTLVFGSWITYNE 180
              * *****:****:***** . * :*****

brugia      NEIKLEFEQAEWVDLSEYAPSSIWDVMDAPAVLVNKRRIEFQVRIRRKTLFYTVVLIIP 213
unc29      NEIKLEFVQAEVDVSEYSASSIWDVIDVPASLVNKRRIEFQVRIRRKTLFYTVVLIIP 240
              ***** ** *:***:*****:*. ** *****

brugia      TVLMAFLSMAVFFLPTDSGEK-----PLMAKYLLLT 244
unc29      TVLMAFLSMAVFFLPTDSGEKITLTISVLLSIVVFLLLVSKILPPTSSTIPLMAKYLLLT 300
              *****

brugia      FVLNVITILVTVIIINVYFRGPTTHRMPKWVRTVFLQ--PKLLCMQRPKPLLRNFTQKNG 302
unc29      FVLNVITILVTVIIINVYFRGPRTHRMPQWVRVVFLLQFLPKLVCMKRPKSASERSAVRSG 360
              ***** *****:***.*** ***:***:***. .. :.*

brugia      HPITAAFPDISELKLNPSIHHLPLCPSAD-----TSAYYPLSPDALRAIDAIE 349
unc29      --MAQLPGVGQFTLSPSAHHLPLCPSADDRTTTIRNTASNETSAYYPLSTDALRAIDAIE 417
              : * :*.:.:.*. ** ***** *****.*****

brugia      <
YIN-HLKQDEEYKMVRDDWKYVAMIIDRLLLYVFFGITVGGTCGILFSAPYVFQGVQDQRA 408
unc29      YITEHLKRDEQHKSFRDDWKYVAMIIDRLLLYVFFGITVGGTCGILFSAPHVFQRIDQQE 477
              **. ***:***:*. *****:*** :*:

brugia      ELQRLIHLYKNGG--- 421
unc29      MLDRLKEKYDTASNIP 493
              **:*** . *....

```

## b) UNC-63

```

brugia      -----QDAGRLIEDLLADYNKLVRPVENSDTLIVRLKLL 36
unc-63      MGPNDHGFAYILIFLLSPPTHANRDLFEDLIADYNKLVRPVSENGETLVVTFKLL 60
              :*.**:***:*****.::.:**:* :***

              >
brugia      SQLLDVHEKNQIMTTNVWLQH-WTDYKWKWNPADYGGVTVLVPSDMIWLPDIVLYS-AD 94
unc-63      SQLLDVHEKNQIMTTNVWLQHSWMDYKLRWDPVEYGGVEVLVPSDTIWLDPVLYNNAD 120
              *****:***** * ***:*:*.**:*** ***** ***:**.* **

brugia      GNYQVSIMTKAKLSPNGTVEWSPPAIYKSMCQIDVEWFPFDIQTCEMKFGSWTYGGLEVD 154
unc-63      GNYQVTIMTKAKLTYNGTVEWAPPAIYKSMCQIDVEFFPFDIQTCEMKFGSWTYGGLEVD 180
              *****:*****: *****:*****:***** * *****:*****

              <
brugia      LQHKD-----GIDLSDYYP SVEWDILGVPGRHLKRYP 187
unc-63      LQHRDKHLEKEIEEDVEGVDGPTKEIVWVVDGIDLSDYYP SVEWDILNVPGRHSLKRYP 240
              ***:* *****.***** **

brugia      CCESPFIDLTYEIRLRKTLFYIVYLIFPIVSISFLTIVLVFYLPSDGGEKISLCNLILIS 247
unc-63      CCESPFIDITYEIHRLRKTLYFTVNLIFPSVGISFLTALVFYLPSDGGEKISLCISILIS 300
              *****:***:***** * *****.*****.*****:*****

brugia      LTIFLLVEIIPSTSLVIPLIGKY-----HFRSPSTHTMPEWT 286
unc-63      LTVFLLVEIIPSTSLVIPLIGKYLLFTMVLVTLSVVVTVVTNLNVHYRSPSTHTMPKWM 360
              **:***** ***:***:**

brugia      KRIFIEFLPKYLLMRPSP-----SSFAS----- 310
unc-63      KRLFVDFLPKYLLMTRPQP PGHHSKPNRKFD SRASTFSIGVNHVLGQNSSELLSPGLNSNR 420
              **:***:***** **.* ***:

brugia      -----FQKELTPVMSAVDSVTFIASHMKDDKNGQQVIEDWKYISVVM DRLFLILFTTAC 364
unc-63      EESSFTLPRDN SPVRS AVESVAYIADHLKNEEDKQVIEDWKYISVVM DRI FLITFTFAC 480
              : :. :** ***:***:**.*:***:..:*****:*** ** **

brugia      MIGSILIILRAPTYLDTTIALA 386
unc-63      AFGTVVIIARAPSIYDNTPALA 502
              :*::** ***:**.* **

```

### **3.4 Discussion**

A bioinformatics approach to investigating the nAChR gene families of parasitic nematodes for which good quality genome sequence is available has revealed a surprisingly small nAChR gene family in *Brugia malayi* and *Trichinella spiralis* compared to *C. elegans*. The close correlation between the nAChR subunits found in both parasites, and the fairly complete and assembled nature of these genome sequences, suggests that this is indeed the full extent of these nAChR gene families. Support for this comes from work using the genome of *Haemonchus contortus*, a clade V nematode quite closely related to *C. elegans*, which has also been carried out in our laboratory. Although the genome sequencing project of *Haemonchus contortus* is much less complete than the parasite genome sequences used here, the majority of the *C. elegans* nAChR subunit genes (~25) were easily identified (Thomas Walsh, personal communication). The results presented here also broadly agree with the annotation accompanying the *Brugia malayi* genome in terms of the number of nAChR subunits present, which was published after the work described in this chapter had been completed. However, the annotators of the *Brugia malayi* genome utilised a rather confusing and inconsistent nomenclature when describing the nAChR subunits, where *C. elegans* gene names were assigned to non-homologous *Brugia malayi* sequences (Ghedini *et al.*, 2007). Figure 3.2 utilises the nomenclature we proposed in Williamson *et al.* 2007, where *C. elegans* gene names are assigned to parasite nAChR subunit sequences which are clearly homologous to *C. elegans* sequences, and a prefix is added to denote the genus and species to which the sequence belongs. This nomenclature has the support of the *C. elegans* nAChR community, and a similar strategy has been used to ensure consistency when annotating the insect nAChR gene families (Andrew Jones and Jonathon Hodgkin, personal communication; Jones *et al.* 2007).

The degree of conservation between putatively homologous nAChR subunits from the different nematode species was variable both within and between nAChR subunits; the ligand-binding motifs and the transmembrane regions were usually quite similar, but the cytoplasmic region was too dissimilar to be identified from genomic sequence in the majority of cases. It would therefore be misleading to attempt to quantify the similarity of homologous nAChR sequences from the regions which could be

identified *in silico*; a more complete comparison would require the amplification and sequencing of all these parasite nAChRs, which is beyond the scope of the work presented here. The most highly conserved nAChR subunit sequences (in terms of the amount of matching sequence which could be easily identified in the parasite genomes) appeared to be *unc-38*, *unc-29* and *unc-63*; this provides a good basis for the design of primers to amplify these subunits from other nematode species. The high degree of conservation suggests these subunits have an essential role in cholinergic signalling in nematodes. When the proposed role of these subunits as components of the levamisole receptor and their changing expression levels in pyrantel resistant hookworms are considered, investigating the function of these nAChR subunits in parasites is clearly key to understanding both sensitivity and resistance to this class of cholinergic anthelmintic (Fleming *et al.*, 1997; Kopp *et al.*, 2008). In contrast to this, the lack of *acr-23* in *Brugia malayi* and *Trichinella spiralis* suggests that if the AAD compounds do in fact have activity against these nematodes, they must act via a different target to the one described from *C. elegans* (Kaminsky *et al.*, 2008).

The findings presented here suggest that either the nematode nAChR gene family underwent a dramatic expansion in the clade V nematodes, or that nAChR genes have been lost from *B. malayi* and *T. spiralis*. Either hypothesis may suggest that this lack of nAChR diversity is linked to the different neurotransmission requirements of a free-living versus a parasitic lifestyle; it should be noted that larvae of many parasitic clade V species must survive outside a host while they develop to an infective stage, whereas transmission from host to host is usually more direct for parasites from clades III and I (Dorris *et al.*, 1999). An alternative explanation for the smaller number of nAChR genes present in these parasites could also be a greater degree of alternative splicing; though insects possess only a small number of nAChR genes, they are known to increase their receptor repertoire by splicing and editing mRNA (Sattelle *et al.*, 2005). No evidence to support this hypothesis has yet emerged from the parasite genomes, but this cannot be entirely ruled out without performing amplification of all the nAChR subunit transcripts generated by the nematodes.

While the dramatic difference in the number of nAChR gene family members between *C. elegans* and the parasites *Brugia malayi* and *Trichinella spiralis* is certainly intriguing, it should be noted that it is currently unknown whether the genomes of these parasites are typical of the clades to which they belong. A more



complete comparison of the gene families of nematodes from different clades cannot be carried out until many more nematode genomes have been successfully sequenced.

## **Chapter 4: Amplification of nAChR subunits from *Ascaris suum***

## **4.1 Introduction**

To investigate the molecular basis of cholinergic anthelmintic sensitivity in a parasitic nematode, the starting point must be to amplify sequences encoding nAChR subunits which are potential components of the relevant receptor. For the model nematode *C. elegans*, a comprehensively sequenced and annotated genome, and the ease with which drug resistant mutants can be produced and maintained *in vitro*, has greatly facilitated the identification of nAChR subunits forming anthelmintic drug targets. For parasitic nematodes, a relative paucity of genomic information, and the difficulties involved in producing or isolating drug resistant worms, makes the task of investigating the molecular basis of drug sensitivity and resistance much more difficult. It should be noted that even when some sequence information is available for a parasite in the form of an EST library, this is often biased towards the most abundant transcripts, and rarely provides more than two or three partial sequences for ligand-gated ion channels. However, several previous studies have succeeded in amplifying a limited number of nAChR subunits from nematode parasites, using two main approaches. Parasite homologues of *unc-38* were obtained from both *Haemonchus contortus* and *Trichostrongylus colubriformis* by constructing and screening genomic DNA libraries (Hoekstra *et al.*, 1997; Wiley *et al.*, 1996). Degenerate PCR using primers based on the amino acid sequence of *C. elegans* nAChR subunits yielded the *Teladorsagia circumcincta unc-38* homologue, and was more recently employed to facilitate amplification of several nAChR sequences from *Ancylostoma caninum* which could not be located by searches of the *A. caninum* sequence database (Kopp *et al.*, 2008; Walker *et al.*, 2001). Both these approaches rely heavily on a high degree of sequence similarity between the sequence being used as a probe or for primer design and the sequence the experiment is designed to obtain; all the parasites mentioned in the previous examples are from clade V and are therefore closely related to each other and to the model nematode *C. elegans*. In the work presented here, it was hoped that degenerate PCR utilising primers designed using the sequence information from the clade III parasite *Brugia malayi* would allow amplification of nAChR subunits from *Ascaris suum* (described in section 3.3). This approach was used to amplify partial sequence of *Ascaris suum unc-29* and *unc-63*.

An existing partial sequence in the GenBank database and the *Ascaris suum* EST database was used to design primers specific to *Ascaris suum unc-38*, and an additional novel nAChR subunit. After partial nAChR subunit sequences had been obtained, RACE techniques were used to amplify the 5' and 3' ends of the sequences (Frohman *et al.*, 1988). RACE techniques have previously been used successfully to obtain full-length transcripts from *Ascaris spp.* (Timinouni & Bazzicalupo, 1997). As a modification to the RACE protocol, the nematode splice leader sequence SL1 was used as the 5' RACE primer. SL1 is a splice-leader element found at the 5' end of the many of nematode mRNA sequences, and has previously been successfully used as a 5' RACE primer for amplification of nematode transcripts (Bektesh *et al.*, 1988; Pape *et al.*, 1999).

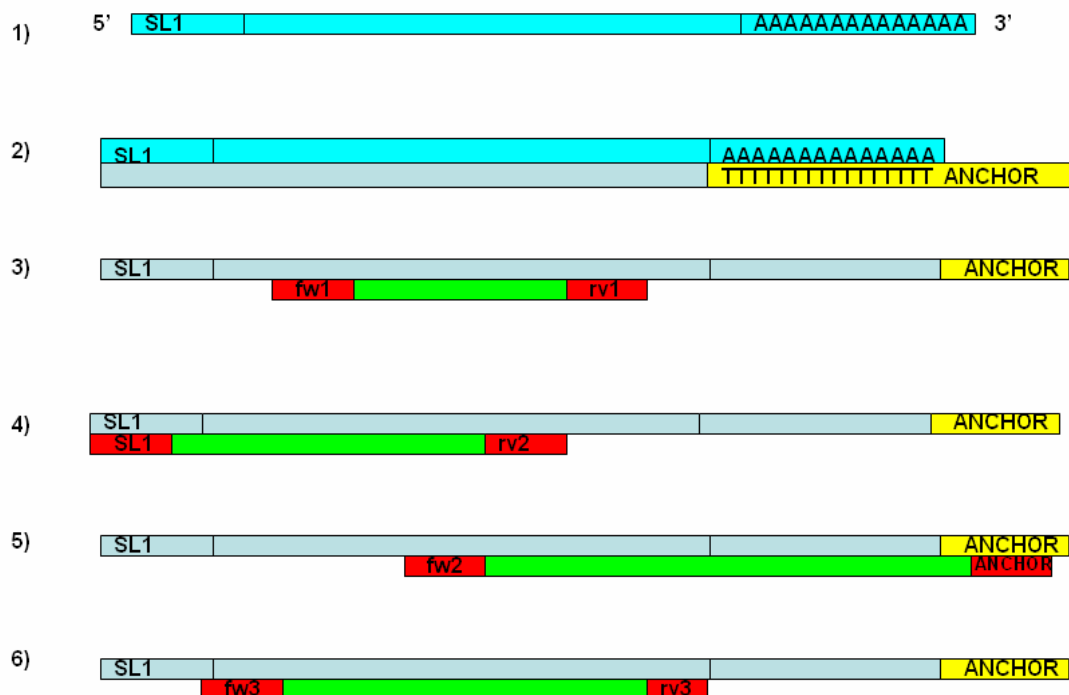
## **4.2 Potential components of the levamisole-sensitive nAChR from *Ascaris suum***

Full length sequences encoding homologues of *unc-38* and *unc-29* were amplified from *Ascaris suum* body wall muscle. The process by which these sequences were obtained is summarised in Figure 4.1. These sequences were deposited in the GenBank database, with the accession numbers EU053155 and EU006073. Figure 4.2 shows the translated sequences of *Ascaris suum* UNC-38 and UNC-29, where some key features are highlighted. Like the homologous *C. elegans* sequence, *Ascaris suum unc-38* encodes a nAChR  $\alpha$  subunit, containing amino acid residues from the principal binding loops A, B and C known to contribute to ligand binding (see Figure 1.4, which is taken from Changeux & Taly, 2008). The loop C motif for both the *C. elegans* and *Ascaris suum unc-38* is Y-X-X-C-C, rather than the more usual Y-X-C-C. This additional loop C residue is a proline known to be involved in the differential sensitivity of nematode and mammalian muscle nAChRs; another residue conferring this differential levamisole sensitivity is a glutamic acid present on loop B, which is again conserved in both *C. elegans* and *Ascaris suum* (Rayes *et al.*, 2004). The *Ascaris suum unc-29* sequence, like the homologous sequence from *C. elegans*, encodes a non- $\alpha$  subunit, lacking the principal binding components but possessing the ligand-binding residues of the complementary binding loops D, E and F (see Figure 1.4, which is taken from Changeux & Taly, 2008). Both *Ascaris suum* UNC-38 and

UNC-29 possess the charged glutamic acid and aspartic acid residues near the base of the second transmembrane domain indicating that they are components of a cation channel (see Figure 1.5, taken from Corringer 2000). This is an important distinction for a nematode nAChR, as *C. elegans* is known to possess acetylcholine-gated anion channels (Putrenko *et al.*, 2005).

### **Figure 4.1: Reverse transcription, amplification, and 5' and 3' RACE**

The schematic shows the different steps involved in obtaining full-length coding sequence for a nAChR subunit sequence. 1) The starting material is mRNA extracted from nematode tissue. Most nematode mRNA has the splice leader sequence SL1 at the 5' end. Also shown is the 3' polyA tail of the mRNA. 2) During reverse transcription, a known anchor sequence is added to cDNA using an oligo dT primer complementary to the polyA tail with this sequence added. 3) A partial sequence of the transcript of interest is amplified by PCR using either degenerate or specific primers. 4) 5' RACE: the known sequence is extended using a forward primer complementary to SL1 and a reverse primer specific for the sequence of interest. 5) 3' RACE: the known sequence is extended using a reverse primer complementary to the anchor sequence added during reverse transcription, and a forward primer specific to the transcript of interest. 6) The whole sequence is now known, and primers to specifically amplify the coding region of the transcript of interest can be designed using the sequence around the start and stop codons.



## **Figure 4.2: The sequences of *Ascaris suum* UNC-38, UNC-29 and UNC-63**

The translated sequences of amplified *Ascaris suum* nAChR subunits which may potentially be components of a levamisole-sensitive nAChR are shown: a) full-length sequence of UNC-38, b) full-length sequence of UNC-29, c) partial sequence of UNC-63. Regions of the sequences are highlighted to display relevant features of the proteins: lower case letters = predicted signal peptide, turquoise = residues necessary for levamisole sensitivity (Changeux & Taly, 2008; Rayes *et al.*, 2004), red = ligand binding residues (Brejc *et al.*, 2001; Changeux & Taly, 2008), green = anionic residues indicative of cation channel specificity (Corringer *et al.*, 2000), yellow = transmembrane domains.

### **a) UNC-38**

mfgllleallwlvvcgtsLTVGNE<sup>DAKRL</sup>YDDL<sup>LMVNYNKHRR</sup>PAMSPHEPVTIKLKLRLSQIIDVHEIDQIMTYSVW  
LKQVWIDKKLSWDPKNYGGVSVLYVPYEMI<sup>N</sup>VPDIVL<sup>N</sup>NNADSNYNITISTKATLHYSGEVTWEPPAIFKSMCQID  
VRWFPPDEQKCHLKFGS<sup>N</sup>TV<sup>SE</sup>DLLVLELLDGEPHYELETNEFGEVDNITIVDDGIDLSDYPSVEWDIMSRVAIR  
RTKN<sup>N</sup>PS<sup>CC</sup>PQSDAVIDIMYYLELRKPLFYTVN<sup>L</sup>VFPCVGISFLTILVFYL<sup>PS</sup>SG<sup>E</sup>KVTLCISILVALTVFFLL  
<sup>L</sup>TEIIPATSISLPLIGKY<sup>LLFTMVMVTL</sup>SVVVTVIS<sup>L</sup>NLHFRTPTTHRMPEWVKWFLKFLPKVLFMRPLADTDD  
TYRRVSQRRGDNCEKVAINYHEHRVSRDIGRALSTSPVDERIQKLYYSPAVVKAFENVCFIAELLKKKDRDDKVDE  
DWKYVAMVLDRL<sup>L</sup>FLLLF<sup>S</sup>FACFIGTVTILL<sup>Q</sup>APTLYDSREIDLQYRPANISTPVTQ

### **b) UNC-29**

mhlp<sup>s</sup>paliivvyv<sup>l</sup>vallragv<sup>l</sup>cSDDEERLMVDVFRGYN<sup>SL</sup>IQPVRLSDTP<sup>II</sup>IVKIALQLVLLIN<sup>VD</sup>ERDQV  
MHTNV<sup>L</sup><sup>L</sup>LLKWHDFQMKWNPVNYGEIQNIRVAPDKVWLPDIVFFNNADGNYEVSFMCNV<sup>L</sup><sup>L</sup>SHK<sup>G</sup>MD<sup>L</sup>W<sup>W</sup>PPAIYK  
SSCIIDVEFFPFDEQ<sup>T</sup>CHLIFGSWTYNENEIKLEFEQAEWDLSEYAPSSIWDVMDAPASLVNKR<sup>S</sup>RIEFQVRIRR  
KT<sup>L</sup>FYTVVLI<sup>I</sup>PTVLM<sup>A</sup>FLSMA<sup>V</sup>FFLPT<sup>SG</sup><sup>E</sup>KMTLTISVLLSIVVFL<sup>L</sup>LV<sup>S</sup>KILPPTSSTIPLMAKY<sup>L</sup>LLTFVLN  
<sup>V</sup>ITILVT<sup>V</sup>IIIN<sup>V</sup>YFRGP<sup>T</sup>THRMPK<sup>W</sup>VRTTFLQVMPKLLCMQRPKQLLRKMAAHAA<sup>Q</sup>QANGAAVATALPGIGEFT  
LNPSAHH<sup>P</sup>FCPSADDSRFHPPSIELTHVDSVRQFERDPMTSAYYPLSADALRAIDAIEYITDHLKQDEEYKMYRDD  
WKYVAMIIDRL<sup>L</sup>LYVFFGITVGGTCGIL<sup>F</sup>SAPYVFQGV<sup>D</sup>QRAELQRLIHLYKNGGEI

### **c) UNC-63 (partial sequence of ligand binding domain only)**

mpclriilwmv<sup>l</sup>slqlkqssNKDASRLFEDLLADYNK<sup>L</sup>VRPVDNNSETLIVRFKLKLSQLLDVHEKNQIMT<sup>N</sup>VW  
LQHSWTDYKLKWN<sup>P</sup>ADYGGVDVLYVPSEMI<sup>N</sup>LPDVVL<sup>N</sup>NNADGNYQVTIMTKAKVSSNGTVEWAPP<sup>A</sup>IYKSMCQID  
VEFFPFDAQTCEMKFGS<sup>N</sup>TV<sup>GG</sup>LEV<sup>D</sup>LKHKDSHKERE<sup>E</sup>LETVLGLDGEYEETVWIVDEGIDLSDYPSVEWDILRV  
PGKRHEKR

Only a partial sequence of *Ascaris suum unc-63* was obtained, which was initially interpreted as being caused by due to mis-priming of the mRNA polyA tail during reverse transcription, as the nucleotide sequence obtained had regions containing many adjacent adenine bases. This possibility was eliminated by altering the reverse transcription primers for a mixture of random hexamers; it was still not possible to amplify any further *unc-63* sequence from *Ascaris* cDNA despite using several different degenerate PCR primers. The sequence obtained did however appear to encode a partial ligand-binding domain of a nAChR  $\alpha$  subunit homologous to *C. elegans unc-63*. This partial sequence is also shown in Figure 4.2.

Tables 4.1, 4.2 and 4.3 show the similarity of the *Ascaris suum* UNC-38, UNC-29 and UNC-63 to other nAChR subunits at the amino acid level. The top 5 most similar sequences returned from a search of the GenBank database using the TBLASTN algorithm are included in each table. In the majority of cases, homologous sequences from other nematode species were returned by the search, in which case only the nematode genus and species is included in the table. Where one of the matching sequences was not from a nematode, the genus and species of the organism concerned, and the designated name for the nAChR subunit using nomenclature appropriate for that species, are included. It should be noted that bias may be introduced into these results by partial sequences, either used as a search query, or as a returned matching sequence from the database. In summary however, there is clearly much greater similarity between homologous nAChR subunits from different nematodes than there is similarity with nAChR subunits from different phyla. As a point of comparison, a similar BLAST search using the *Ascaris suum* sequences was performed but the results restricted to human nAChR sequences. These generally showed roughly 40% identity to the nematode subunits, though surprisingly, UNC-38 and UNC-63 appeared slightly more similar to human  $\alpha 6$  and  $\alpha 4$ , and UNC-29 to human  $\beta 2$ , than to the human muscle nAChR subunits. The similarity of UNC-29 to  $\beta 2$  is also apparent in the conserved ligand binding residues (Brejc *et al.*, 2001).



**Table 4.1: Similarity between *Ascaris suum* UNC-38 and the 5 most similar nAChR subunits returned by a search of the GenBank database using the TBLASTN algorithm**

Species	Percentage identity	Accession number
<i>Haemonchus contortus</i>	86%	U72490
<i>Caenorhabditis elegans</i>	80%	NM_059071
<i>Ancylostoma caninum</i>	77%	EU852579
<i>Brugia malayi</i> (partial sequence)	78%	XM_001901151
<i>Onchocerca volvulus</i> (partial sequence)	73%	L12543

**Table 4.2: Similarity between *Ascaris suum* UNC-29 and the 5 most similar nAChR subunits returned by a search of the GenBank database using the TBLASTN algorithm.**

Species	Percentage identity	Accession number
<i>Brugia malayi</i>	89%	XM_001898590
<i>Haemonchus contortus</i>	81%	EU006786
<i>Ancylostoma caninum</i>	81%	EU852580
<i>Caenorhabditis elegans</i>	77%	NM_059998
<i>Caenorhabditis briggsae</i>	79%	XM_001668341

**Table 4.3: Similarity between *Ascaris suum* UNC-63 and the 5 most similar nAChR subunits returned by a search of the GenBank database using the TBLASTN algorithm.**

Species, nAChR subunit name (if not a clear homologue from another nematode species)	Percentage identity	Accession number
<i>Haemonchus contortus</i>	81%	EU006787
<i>Ancylostoma caninum</i>	81%	EU852581
<i>Caenorhabditis elegans</i>	77%	NM_059132
<i>Brugia malayi</i> (partial sequence)	79%	XM_001899057
<i>Bemesia tabaci</i> $\alpha$ -3 subunit	57%	AJ880081

### **4.3 A novel nAChR subunit from *Ascaris suum***

An additional nAChR subunit not thought to be a component of the levamisole-sensitive neuromuscular nAChR was also amplified from *Ascaris suum*, but was amplified from head tissue rather than body wall muscle. A partial sequence of this subunit was present in the *Ascaris* EST database, which is surprising as generally only transcripts expressed at high levels are present in amongst such data. The bioinformatics analysis reported in chapter 3 also found a homologous sequence in the genome of *Brugia malayi*, which with the agreement of the annotators of the *C. elegans* nAChRs was designated *acr-26*. A remarkable feature of the *acr-26* sequence is that it is clearly not homologous to any known *C. elegans* nAChR subunit, and is instead most similar to a type of nAChR subunit present in the pond snail *Lymnaea stagnalis* (van Nierop *et al.*, 2006). Table 4.4 shows sequence similarity between *Ascaris suum* ACR-26 and other nAChR subunits present in the GenBank database. The closest match is an ACR-26 homologue from *Haemonchus contortus* which was also amplified in our laboratory (accesssion number EU006791; Thomas Walsh, unpublished). This finding discounts the possibility that the new subunit is specific to the clade III nematodes, and instead raises the intriguing possibility that it is specific to parasites from both clades V and III. If the search of similar nAChR subunits is restricted to human nAChR subunits,  $\alpha 7$  and  $\alpha 10$  are most similar to ACR-26 (37%

and 36% identity). The ACR-26 sequence is shown aligned against the a mammalian  $\alpha 7$  sequence in Figure 4.3. The choice of  $\alpha 7$  for the alignment is based on the fact that all the nAChR sequences more similar to ACR-26 represent extremely obscure subunits about which little is known; in addition to this, alignment against  $\alpha 7$  shows that ACR-26 also appears to possess the ligand binding residues of the complementary binding loop D (see Figure 1.4 taken from Changeux & Taly 2008; also the alignment from Brejc *et al.*, 2001) suggesting that it may be capable of forming a homomeric receptor.

**Table 4.4: Similarity between *Ascaris suum* ACR-26 and the 5 most similar nAChR subunits returned by a search of the GenBank database using the TBLASTN algorithm**

Species, nAChR subunit name (if not a clear homologue from another nematode species)	Percent identity	Accession number
<i>Brugia malayi</i> (partial sequence)	65%	XM_001901191
<i>Haemonchus contortus</i>	70%	EU006791
<i>Lymnaea stagnalis</i> subunit G	63%	DQ167350
<i>Lymnaea stagnalis</i> subunit D	46%	DQ167347
<i>Apis mellifera</i> $\alpha$ -6 subunit	39%	DQ026035

**Figure 4.3: Alignment of the novel nAChR subunit ACR-26 from *Ascaris suum* with the human neuronal  $\alpha 7$  subunit**

The novel nAChR subunit ACR-26 from *Ascaris suum* has been aligned against the human  $\alpha 7$  subunit to demonstrate similarities in the ligand binding residues. Like  $\alpha 7$ , ACR-26 has ligand binding residues belonging to both the principal binding loops characteristic of  $\alpha$  subunits (coloured red, see Figure 1.4; annotated from Changeux & Taly, 2008, and Brejc *et al.*, 2001), and residues from the complementary binding loop D (coloured turquoise, see Figure 1.4; annotated from Changeux & Taly, 2008, and Brejc *et al.*, 2001). Also shown are residues conferring cation specificity (coloured green; annotated from Corringer 2000, see Figure 1.5) and predicted transmembrane regions (yellow).

```

ACR26      MATRRRELILLUFIHGVDVVLADSSITDLSEIDGTATDEQKLLYHLLRQYEKAVRPVRN
a7          -----MRCSPGGVWLALAAS----LLHVSLQGEFQRKLYKELVKNNPLERPVAN
              :      * * : . * *      * . : .      : : * *      . * : : : :      * * * *

ACR26      ASHTVTVKLGMTMTNIFEMDEKNQVLTINVWLDQEWKDELLRWDPKEFGGIQSIRIPCDL
a7          DSQPLTVYFSLSLQIMDVDEKNQVLTNTNIWLSWTDHYLQNNVSEYPGVKTVRFPDQG
              * : . : * *      : . : :      : : : : * * * * * *      * : * *      . * :      : : * : : : : * * .

ACR26      ILLPDIIVLNNADDYTAGYMRSRAMVFYDGTVFVPPPTQLRSTCKIDVTYFPFDSQHCAL
a7          IWKPDILLNNSADERFDATFHTNVLVNSSGHCQYLPPIGFKSSCYIDVRWFPPFDVQHCCKL
              * * * * : * * * * * :      .      : : : : *      . *      :      * *      : : * *      * * * * * * *

ACR26      KFGSNTYHGFQVDITNRSDNVDLSNYVVSGEFDLVRVHQKRRVVKITCCPEPYPDVTFFI
a7          KFGSNTSYGGWSLDLQMQ--EADISGYIPNGEWDLVGIPGKRSEFYECCKEYPDVTFITV
              * * * * : * * : : * :      : . : * : * :      . * : * * * :      * *      * * * * * * * :

ACR26      HIRRKTLYYLYNVVFPMMMSVLTLLVFLLPDSSGKIALGITVLLAFSVFVLAIAEKMP
a7          TMRRTLYYGLNLLIPCVLISALALLVFLLPDSSGKISLGITVLLSLTVFMLLVAIMP
              : * * : * * *      * : : : * : : * : * * * * * * * * * * : : * * * : * * * *

ACR26      ETSDSMPLIGIYLTVMAMTSVSVVMTVMVLNFHHRGPFNQAVPKVWVHRLVLNRLRRALC
a7          ATSDSVPLIAQYFASTMIIVGLSVVTVIVLQYHYHDPDGGKMPKWTRVILLNWCWFLR
              * * * * : * : :      . * : . : * * : * * : * : : *      .      : * * : : * *      *

ACR26      MRLPYTG-----WKDNGFCSANGMTKTISIGLAMDDFNVDSEFELDNQFLQMQETELVN
a7          MKRPGEDKVRPACQHKQRRCSLASVEMSAVVPPASNGNLLYIGFRGLDGVHCVPPTDPSG
              * :      *      .      : :      * *      . :      :      .      . :      * *      :      .      : :      *      .

ACR26      VDDAAALRKKKCRPNELHS-----RLKTLQVLIRRQEMEDLYQTLANEWRQVA
a7          VVCGRMACSPTHDEHLLHGGQPPEGDPDLAKILEEVRYIANRFRQDESEAVCSEWKFAA
              *      .      .      .      : * *      .      : : * : : :      . *      . : *      : : : . * * : *

ACR26      QVIDRLFWVFLVCTVIITLILLIIPAVHRSMESDVDFDESLYGLH
a7          CVVDRLCLMAFSVFIICTIGILMSAPNFVEAVSKDFA-----
              * : * * :      . *      * * * * : * :      *      .      . : : . * .

```

## **4.4 Real-time PCR**

Real-time PCR was performed to ascertain whether relatively low expression levels of *Ascaris suum unc-63* were causing difficulty in amplifying the full-length transcript. This technique also allowed a comparison to be made of expression levels of *unc-38*, *unc-29* and *unc-63* in tissue taken from *Ascaris suum* head and body wall. Head and muscle tissue were taken from 3 different individual nematodes and the RNA extracted. Reverse transcription reactions were performed on each batch of RNA, using the same amount of total RNA in the reaction each time. A reference gene expressed at the same levels in both head and muscle tissue was also amplified alongside the nAChR subunit genes to ensure the amplification in each reaction was comparable. Two replicates were performed for each amplification reaction from each batch of cDNA. Examples of amplification plots are shown in Figure 4.4. The real-time PCR data is also reported as mean critical threshold (Ct) values in Table 4.5; critical threshold values indicate the number of PCR cycles necessary before the amplification of a transcript reaches the detection threshold.

A comparison of relative abundance of the nAChR subunits in tissue taken from *Ascaris suum* head and body wall showed that there is very little difference in expression levels between these different regions of the nematode. This finding can only be preliminary however, as only very crude dissections were performed to obtain the tissue used this experiment. The head preparations would have contained pharyngeal tissue, sensory amphids, and the nerve ring in addition to muscle tissue; the body wall muscle would also have contained motoneurons and regions of the dorsal and ventral nerve cords. A more thorough comparison of nAChR subunit expression levels in different tissues would therefore require a more detailed dissection which separated the muscle from the nervous system and other tissues, and would require more fresh parasite tissue than was available during this investigation.

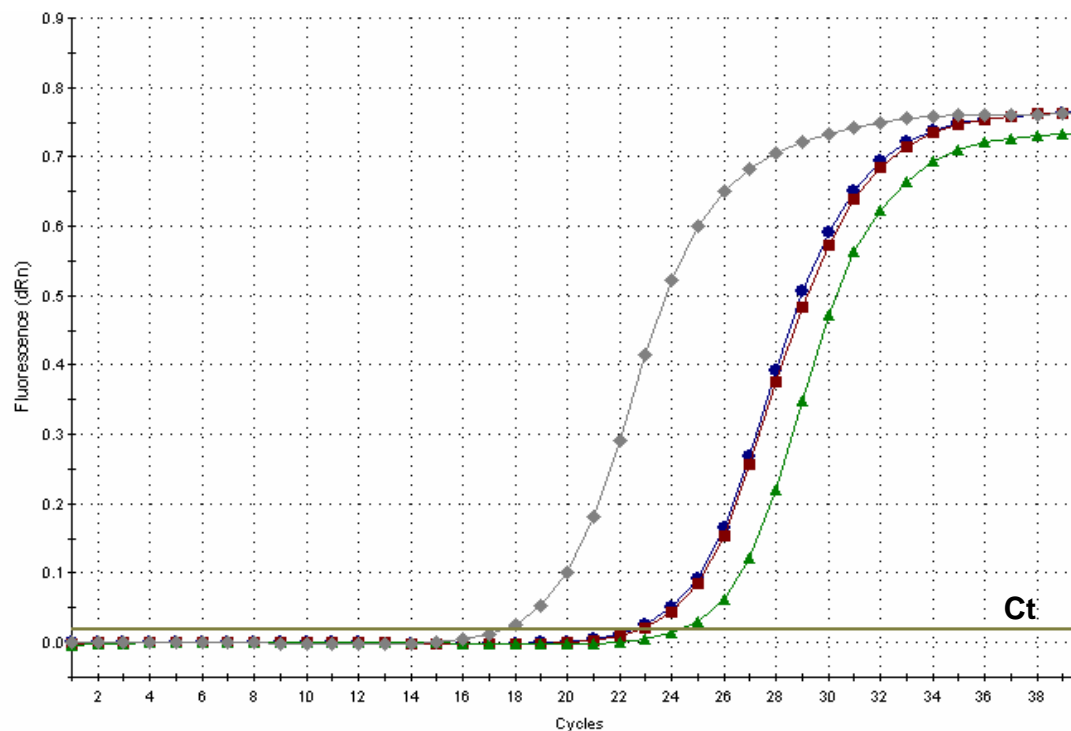
When the relative abundance of the different nAChR subunits were compared, amplification of *unc-63* (as shown by the Ct values in Table 4.5) occurred consistently later than amplification of *unc-38* and *unc-29*. The Ct values suggest that *unc-38* and *unc-29* transcripts are perhaps equally abundant, whereas *unc-63* is expressed at a slightly lower level. However, the relative expression level of *unc-63* did not appear

low enough to be the major difficulty underlying the amplification of a full-length *unc-63* transcript.

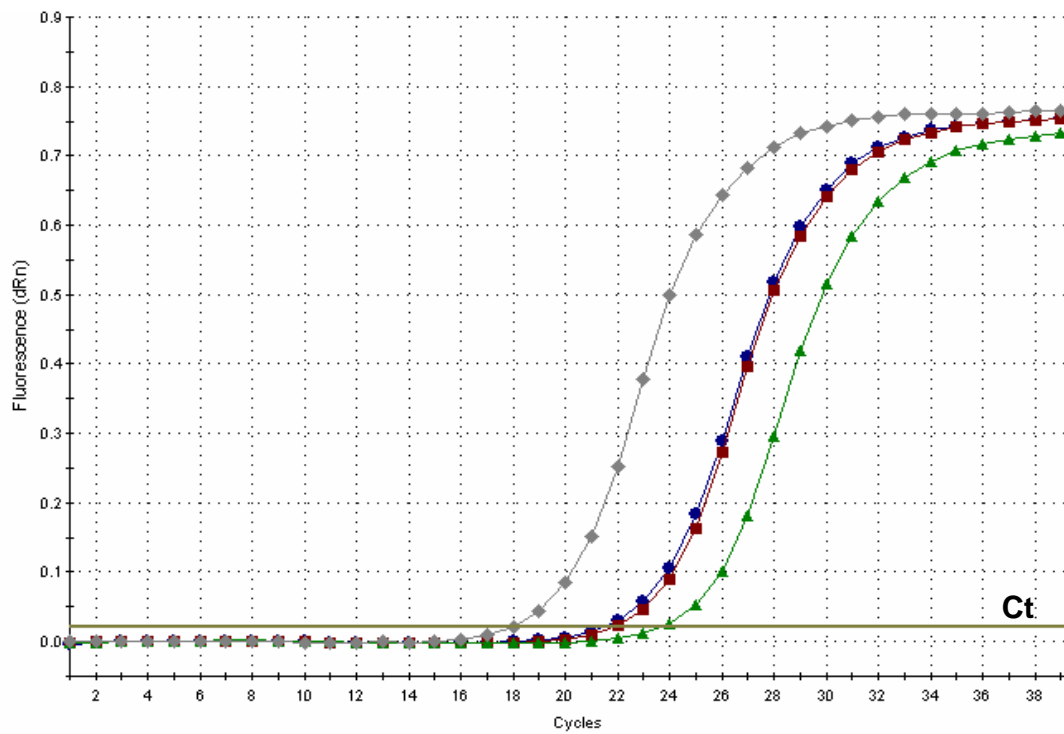
**Figure 4.4: Amplification plots showing the relative abundance of *Ascaris suum* *unc-38*, *unc-29* and *unc-63* in tissue taken from a)head and b)body wall.**

Amplification plots showing amplification of the reference gene  $\beta$ -tubulin (grey) and the nAChR subunit sequences *unc-38* (blue), *unc-29* (red) and *unc-63* (green) by real-time PCR. The Y-axis shows increasing fluorescence as more DNA is amplified. The X-axis shows the number of PCR cycles. The horizontal line near the bottom of the graph labelled Ct indicates the critical threshold above which amplification is detected.

a) head



b) body wall



**Table 4.5: Critical threshold (Ct) values for real-time PCR amplification of reference gene  $\beta$ -tubulin and nAChR subunits *unc-38*, *unc-29* and *unc-63* from *Ascaris suum* head and body wall tissues**

	head	body wall
$\beta$ -tubulin	18.17 +/- 0.51	17.98 +/- 0.04
<i>unc-38</i>	23.51 +/- 0.84	23.19 +/- 0.83
<i>unc-29</i>	24.16 +/- 0.80	23.82 +/- 1.01
<i>unc-63</i>	25.94 +/- 0.76	25.23 +/- 0.79

## **4.5 Discussion**

In the course of this investigation, 3 full-length nAChR subunit sequences have been cloned from *Ascaris suum* cDNA; two of these were clear homologues of the *C. elegans* nAChR subunits *unc-38* and *unc-29*, and are therefore good candidates for components of a levamisole-sensitive nAChR (Fleming *et al.*, 1997). The additional full-length subunit, named *acr-26*, has no clear homologue in *C. elegans* but has been shown to be present in the parasites *Brugia malayi* and *Haemonchus contortus*, demonstrating that this subunit may be worthy of further investigation as a potential new anthelmintic target. The amplification of a partial sequence of *unc-63* from *Ascaris suum* supports the findings described in chapter 3 that *unc-63* appears to be present in other parasitic nematodes; this is further supported by the amplification of *unc-63* from *Ancylostoma caninum* by McCarthy and colleagues. However, the failure to successfully extend the partial sequence to full length using either additional degenerate primers designed on conserved regions of other *unc-63* sequences, or using a primer linked to the polyA tail of the mRNA during the reverse transcription, cannot be satisfactorily explained by the work presented here. Although *unc-63* appears to be expressed at lower levels than *unc-38* and *unc-29*, it is clearly detectable by PCR. It is however a possibility that in *Ascaris suum*, the *unc-63* transcript may be alternatively spliced, or simply truncated; such matters cannot be resolved until the genome sequence of *Ascaris suum* is completely sequenced, assembled and available to search.

The amplification of cDNA sequence from *Ascaris suum* encoding these nAChR subunits provides a basis for further investigations into the molecular biology underlying the pharmacology of *Ascaris suum* nAChRs, in particular the basis of levamisole and pyrantel sensitivity. If *Ascaris suum* populations resistant to these drugs were to become available, the sequence of *unc-38* and *unc-29* could easily be checked for polymorphisms which may underly this differential drug sensitivity. In addition to this, if drug resistance was caused by a change in the expression levels of nAChR subunits, we have demonstrated that expression levels of *unc-38*, *unc-29* and *unc-63* from *Ascaris suum* can be quantified using real-time PCR. Such a difference in expression levels was found between hookworms resistant and susceptible to



pyrantel, and studies such as these can provide the basis for diagnostic assays to assess the level of drug resistance in a parasite population (Kopp *et al.*, 2008; Walsh *et al.*, 2007).

## **Chapter 5: Immunohistochemical localisation of *Ascaris suum* UNC-38, UNC-29 and UNC-63**

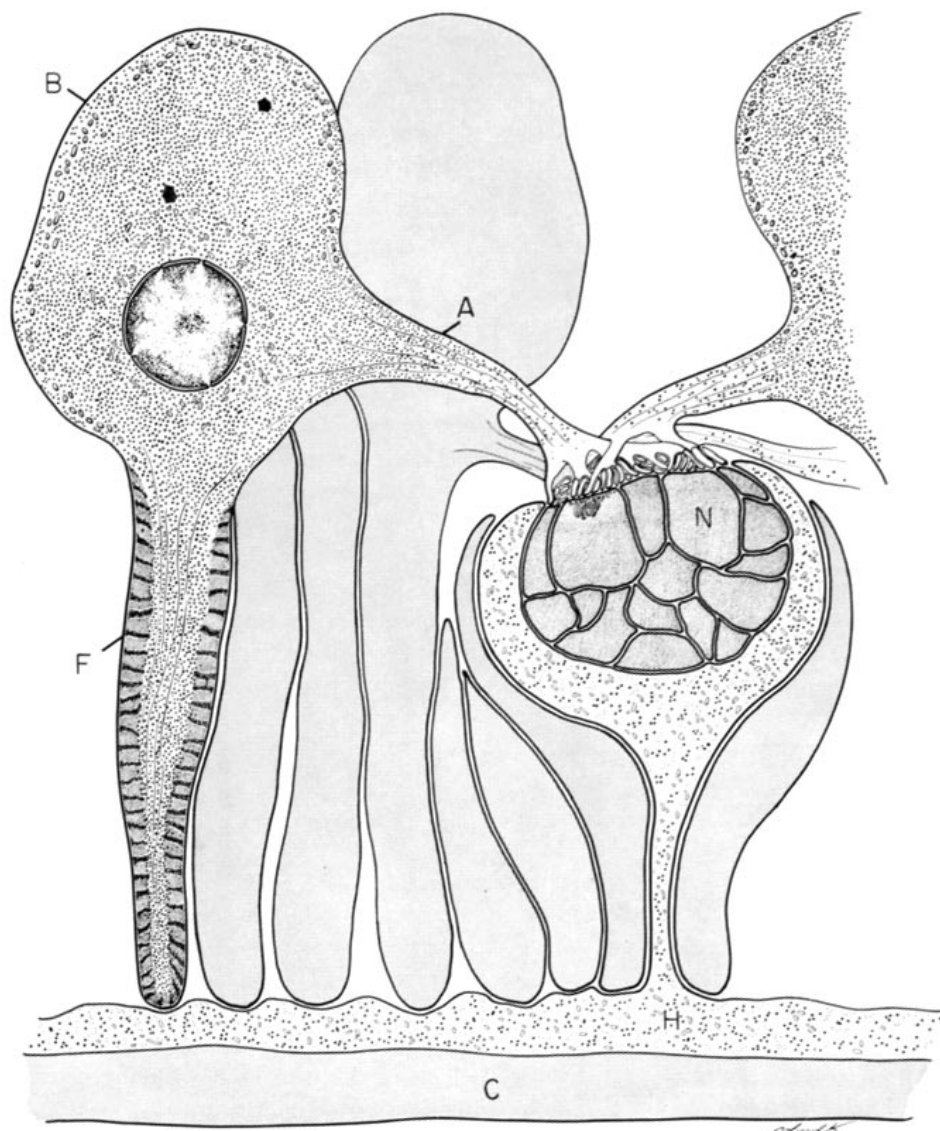
## **5.1 Introduction: Ascaris muscle cells and the nematode neuromuscular junction**

The structure of muscle cells and the neuromuscular junction of nematodes differs markedly from that of vertebrates. A detailed study of the structure of *Ascaris* muscle cells and the neuromuscular junction was performed by Rosenbluth (1965), and is summarised in Figure 5.1. A typical muscle cell has 3 distinct regions: the contractile region, the "bag" or "belly" region of the cell containing the nucleus and glycogen stores, and a muscle arm which extends towards and contacts the nerve, forming the neuromuscular junction (Rosenbluth, 1965). It has since been shown, however, that the size of the bag region can be much reduced in some muscle cells, and that the number of muscle arms extending from a cell is also variable (del Castillo *et al.*, 1989; Stretton, 1976). In the region nearest to the nerve cord the muscle arms branch into very fine processes, which contact the muscle arm processes of adjacent muscle cells (Rosenbluth, 1965). Adjacent muscle cells have been shown to be electrically coupled via gap junctions connecting the muscle arm processes in this region, which is often termed the syncytium (de Bell *et al.*, 1963). The presence of cholinergic receptors in *Ascaris* muscle tissue is well documented, as both muscle contractions and electrophysiological responses have been observed in response to cholinergic agonist application (Baldwin & Moyle, 1949; Colquhoun *et al.*, 1991).

In order to investigate whether the nAChR subunits UNC-38, UNC-29 and UNC-63 were localised to the *Ascaris suum* the muscle cell membrane, polyclonal antibodies were raised against subunit-specific synthetic peptides and used to carry out indirect immunofluorescent labelling of *Ascaris* muscle cells.

**Figure 5.1: The structure of the muscle cell and neuromuscular junction from *Ascaris suum* (from Rosenbluth 1965)**

The muscle cell of *Ascaris suum* is divided into 3 distinct regions: the contractile region (F), the bag or "belly" of the cell containing the nucleus and glycogen stores (B), and the muscle cell arm (A) which branches into finer processes as it approaches the nerve cord (N) to form the neuromuscular junction. This differs from the vertebrate neuromuscular junction in that the muscle cells form processes which extend out to contact the nerve, rather than processes from the nerve cell extending out into the muscle.



## **5.2 Choice of peptides for antisera production and ELISA results**

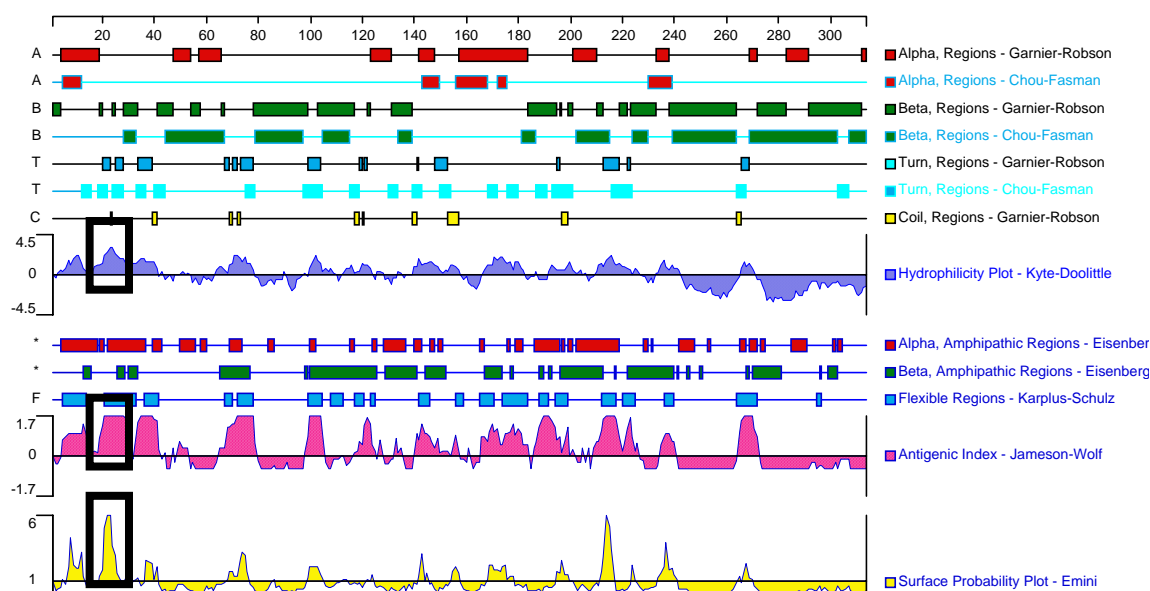
In order to determine regions of the nAChR subunits suitable for use as peptides for the production of antisera, the translated N-terminal domain sequence of UNC-38, UNC-29 and UNC-63 was analysed using ProteAN software. The results of this are shown in Figure 5.2.1. The chosen peptides were predicted to be hydrophilic, antigenic, and had a high probability of being present on the surface of the protein in its folded conformation. The selected peptides were located at the extreme N-termini of the subunits just after the predicted signal peptide cleavage site, a strategy successfully used previously to raise antisera against nematode glutamate-gated chloride channels (Delany *et al.*, 1998).

The antisera were affinity purified, and enzyme-linked immunosorbent assays were performed to ensure that the antibodies had activity against the peptides used for antisera production. The results are shown in Figure 5.2.2, and confirm that the terminal antisera and affinity purified antibody solutions all show a greater titre of antibodies which react against the peptides used for antisera production than the pre-immunw serum. The affinity purified antibody solutions also appeared to show less non-specific reactivity at greater dilutions than the terminal antiserum. The ELISA results were also used to determine an appropriate dilution of antibody for use in the subsequent indirect immunofluorescence procedures.

**Figure 5.2: ProteAn analysis of the sequences of *Ascaris suum* UNC-38, UNC-29 and UNC-63**

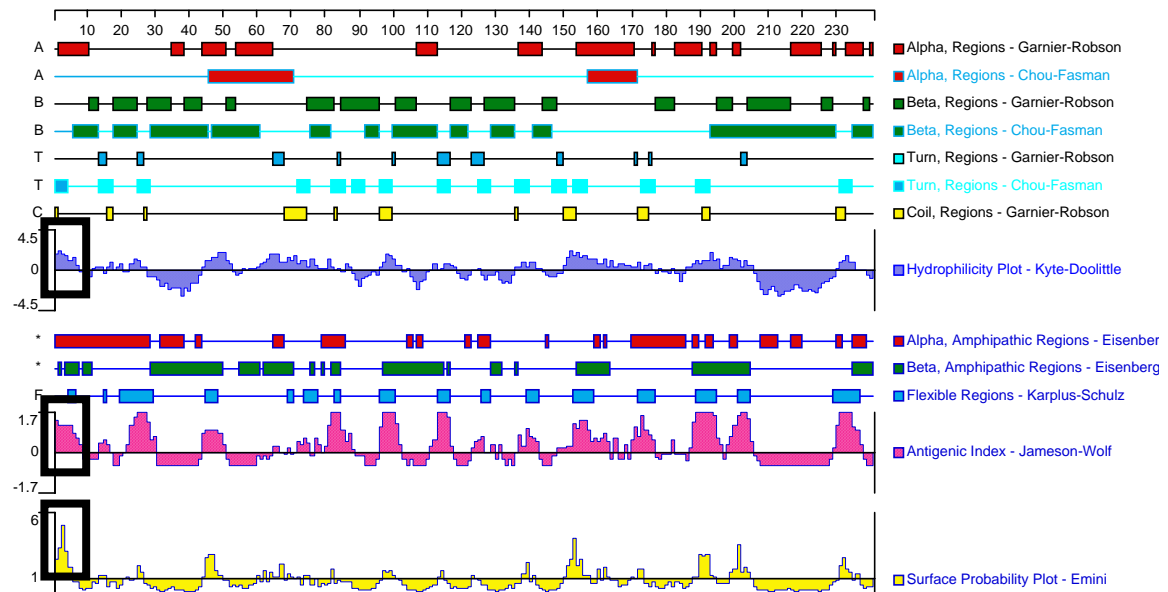
The ProteAn analysis software was used to predict the properties of the peptide sequence of the N-terminal domains of the *Ascaris suum* nAChR subunits. The relevant properties for use in antisera production are hydrophilicity, antigenicity and high surface probability, and these properties for the chosen peptide are outlined with a box. Below the ProteAn output the N-terminal subunit sequence is shown, with the predicted signal peptide removed, and the peptide chosen for antisera production highlighted.

### a) UNC-38



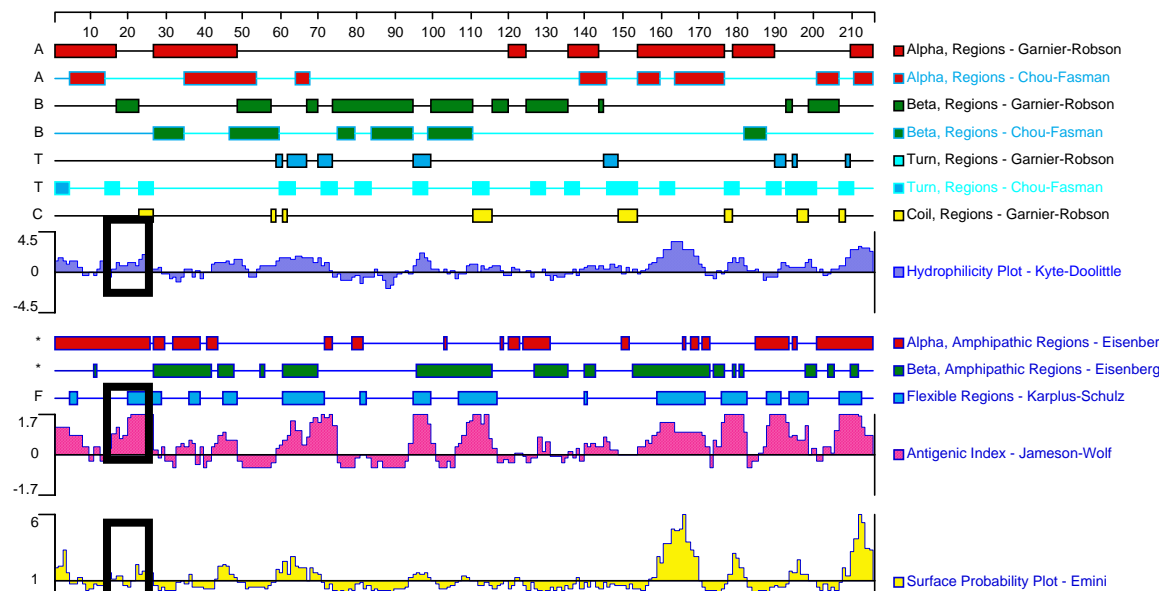
LTVGNE**DAKRLYDDLMVNYNK**HRRPGNVTTTRTCHNQAKTSPFQIIDVHEIDQIMTCSVWLKQVWIDKKL  
 SWDPKNYGGVSVLYVPYEMIWVPDIVLYNNADSNYNITISTKATLHYSGEVTWEPPAIFKSMCQIDVRW  
 FPFDEQKCHLKFGSWTYSEDLLVLELLDGEPHYLETNEFGEVDNITIVDDGIDLSDYYPSVEWDIMSR  
 VAIRRTKNYPSCCPQSDAYIDIMYYLELRKPLFYTVNLVFPVCGISFLTILVFYLPSDSGEKVTLCIS  
 ILVALTVFFLLLTEIIPATSISLPLIGKYLLFTMVMV

## b) UNC-29



SDDEERLMVDVFRGYN SLIQPVRNLS DTP IIVKIALQLVLLINVD ERDQVMHTNVWLT LKWHDFQMKWN  
 PVNYGEIQNIRVAPDKVWLPDIVL FNNADGNYEVSFMCNVVINHKG DMLWVPPAIYKSSCIIDVEFFFPF  
 DEQTCHLIFGSWTYNENEIKLEFEQAEWVDLSEYAPSSIWDVMDAPASLVNKR SRIEFQVRIRRKTLFY  
 TVVLI IPTVLM AFLSMAVFFLPTDSGEKMTLTI

## c) UNC-63

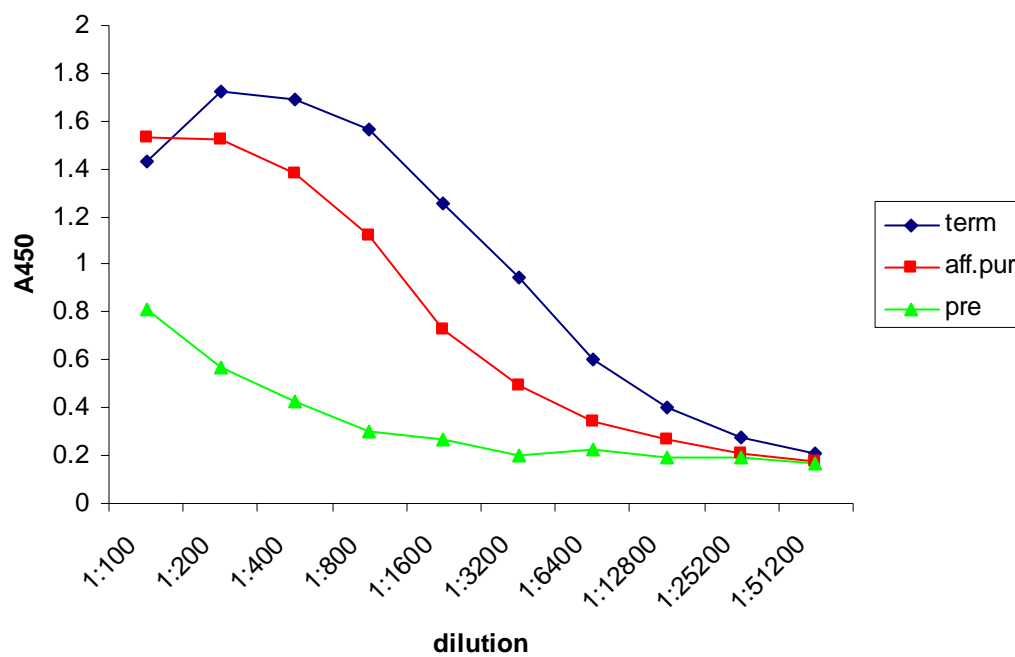


NKDASRLFEDLLADYNKLVRPVDNNSETL IVRFKLKLSQLLDVHEKNQIMTTNVWLQHSWTDYKLKWNP  
 ADYGGVDVLYVPSEMIWLPDVVLYNNADGNYQVTIMTKAKVSSNGTVEWAPP AIYKSMCQIDVEFFFPD  
 AQTCEMKFGSWTYGGLEVDLKHKDSHKERELEETVLGLDG EYEETVWIVDEGIDLSDYPSVEWDILRV  
 PGKRHEKR

**Figure 5.3: ELISA results for antibodies against *Ascaris suum* UNC-38, UNC-29 and UNC-63**

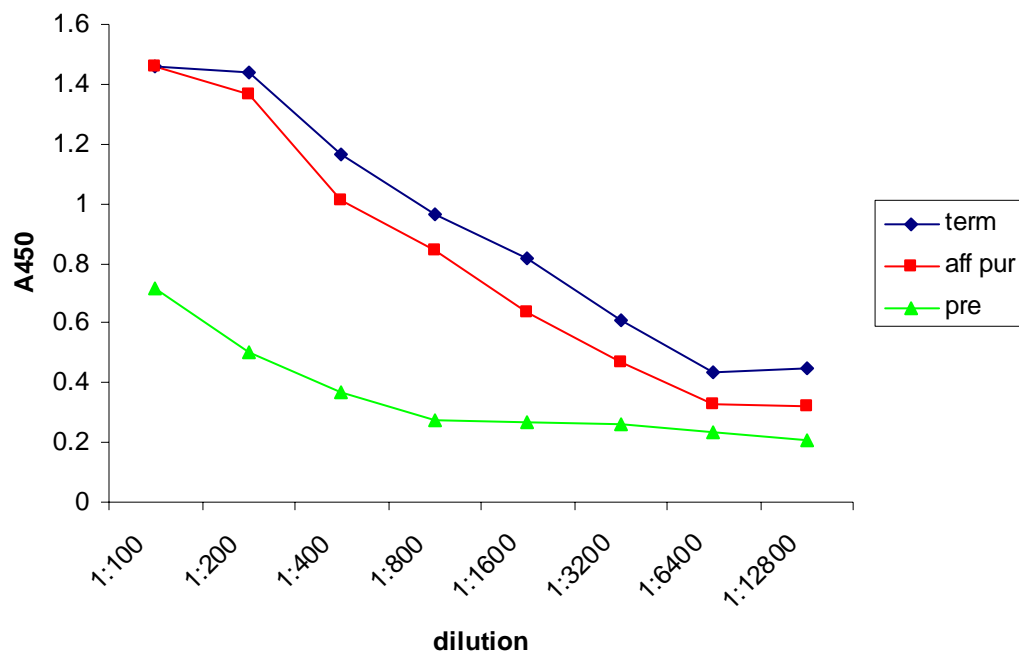
The ELISA results show a comparison of the reactivity of the terminal antisera, pre-immune antisera and affinity purified antibodies against the peptides used for antisera production.

**a) Antibody against UNC-38**

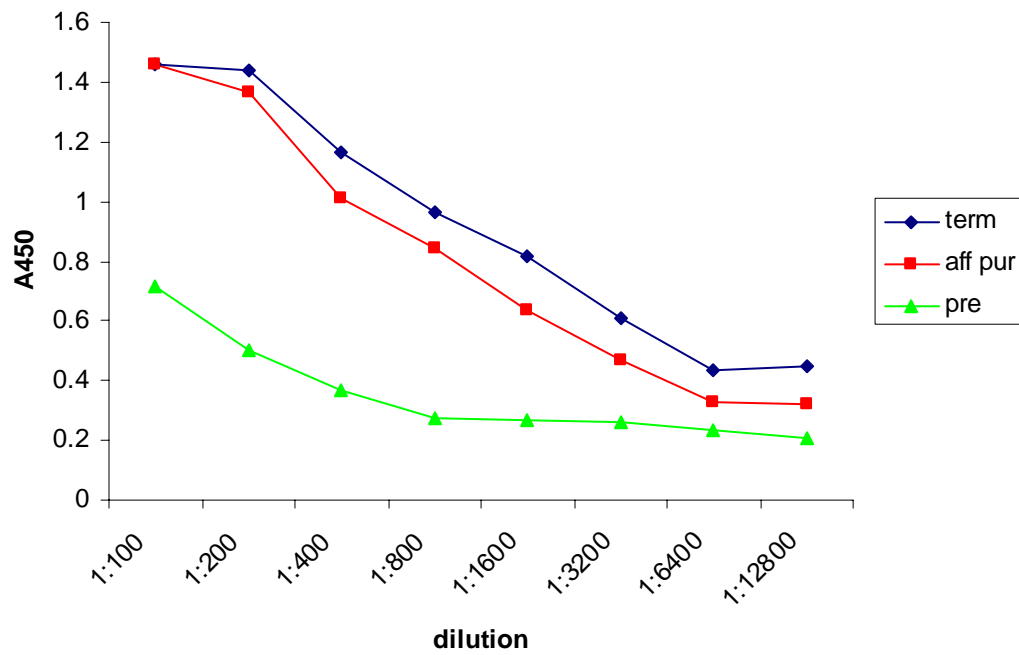




**b) Antibody against UNC-29**



**c) Antibody against UNC-63**



### **5.3 Indirect Immunofluorescence**

Muscle cells and cuticle-mounted nerve cords were prepared by collagenase digestion of fresh *Ascaris suum* adult females as described in section 2.4.4 (Johnson & Stretton, 1985). The material was then fixed and sequentially incubated with primary and secondary antibody solutions as described in section 2.4.5 (Brownlee *et al.*, 1993). Negative controls were used to demonstrate that the antibody labelling was specific to the nAChR subunits; these are shown in Figure 5.3.1. The negative controls were pre-absorbed primary antibody, normal goat IgG, and secondary antiserum alone. The negative controls all showed only very low levels of immunofluorescence consistent with very little non-specific antibody labelling. Unfortunately the microscope used did not have a phase-contrast feature which would have allowed clear images of the specimens to be obtained using visible light.

Initially, *Ascaris suum* muscle cells, and strips of cuticle with attached nerve cord, were incubated with antibody against UNC-38. The purpose of this was to investigate whether the nAChRs containing this subunit were neuromuscular or neuronal. The results of this are shown in Figure 5.3.2. UNC-38 is clearly localised to the muscle cell membrane, and is present over the entire muscle cell surface. The neuronal tissue itself was not labelled with the antibody against UNC-38, but the nerve-cord preparation did reveal details of the muscle arm processes contacting the nerve cord to form the neuromuscular junctions.

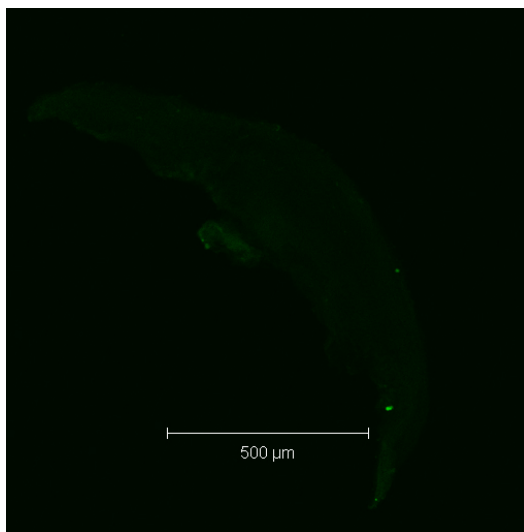
Muscle cells were then incubated with primary antibodies against both UNC-38 and UNC-29, followed by secondary antibodies conjugated to different fluorescent labels, to ascertain whether UNC-38 and UNC-29 co-localised. The results of this are shown in Figure 5.3.3. Both UNC-38 and UNC-29 were localised to the muscle cell membrane, and clearly co-localised, especially in the region of the muscle cell arm. At high magnification, the co-localised fluorescence has a punctate appearance, which may represent receptor clusters in the distal region of the muscle cell arm.

Finally, muscle cells were incubated with antibodies against UNC-63, the results of which are shown in Figure 5.3.4. UNC-63 is also clearly localised to the muscle cell arms of *Ascaris suum*. However, attempts to perform co-localisation experiments using antibodies against UNC-63 and UNC-29 together were unsuccessful, with only the UNC-29 labelling being visible (results not shown). Possible reasons for this are discussed in section 5.4.

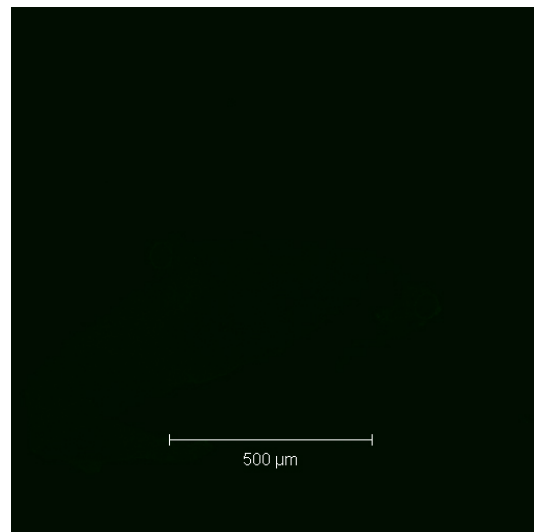
**Figure 5.4: Confocal microscope images of negative controls for indirect immunofluorescent labelling of *Ascaris suum* muscle cells**

In the absence of specific primary antibody, very little fluorescence was observed on muscle cells after incubation with a 1:200 dilution of FITC conjugated secondary antiserum.

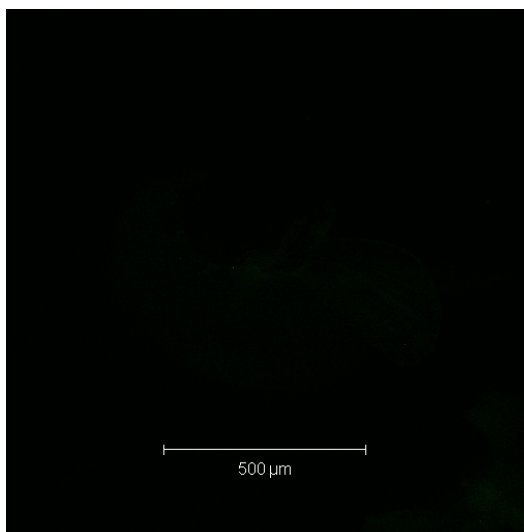
**a) Normal goat IgG**



**b) Pre-absorbed primary antibody**



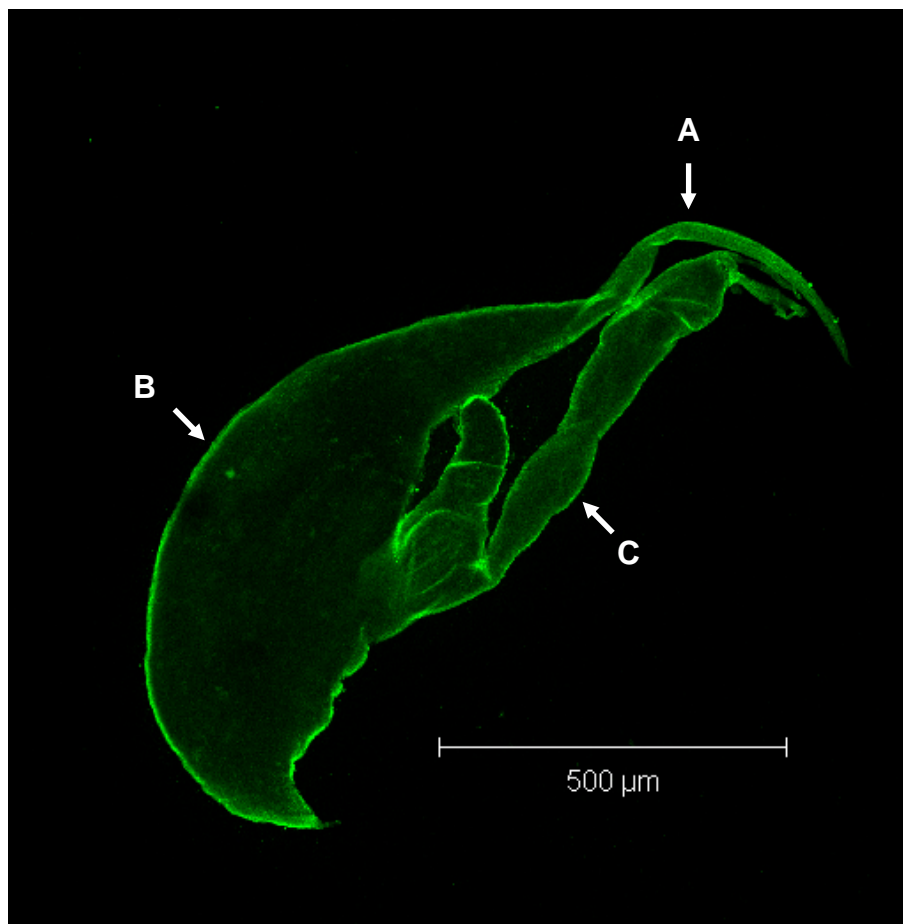
**c) Secondary antiserum only**



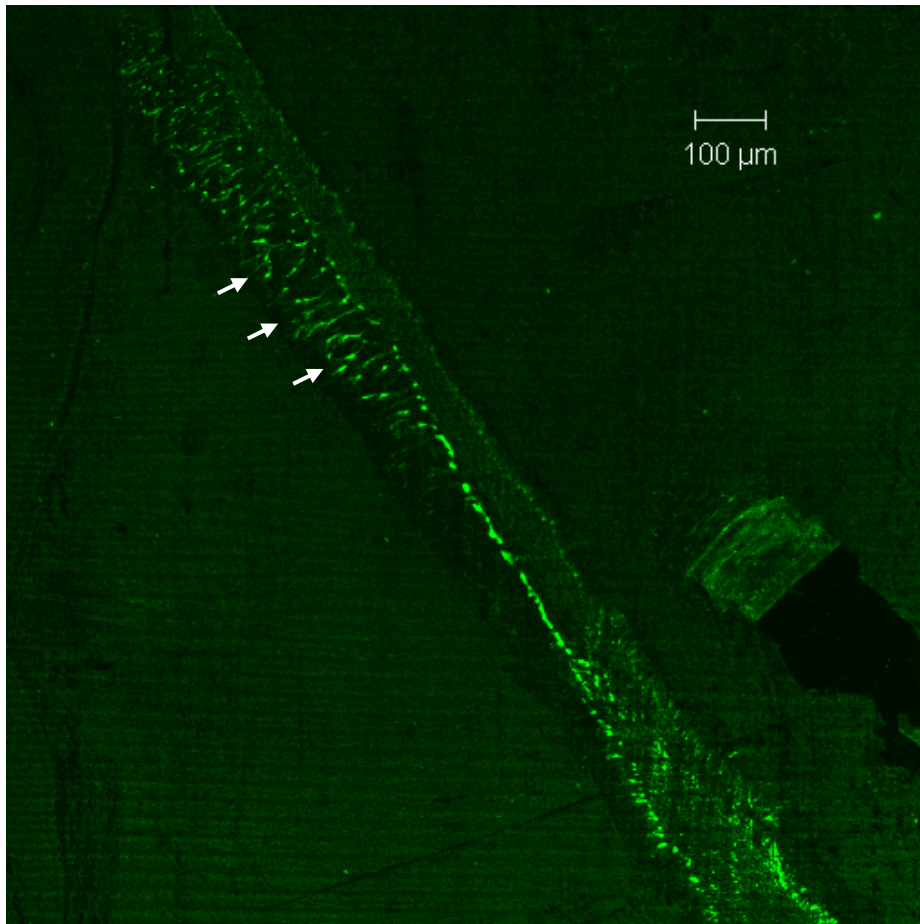
**Figure 5.5: Indirect immunofluorescent labelling of *Ascaris suum* UNC-38**

Confocal microscope images showing indirect immunofluorescent labelling of *Ascaris suum* muscle with primary antibody against UNC-38 (affinity purified from goat serum, used at 1:100 dilution) and FITC conjugated secondary antibody (rabbit anti-goat, used at 1:200 dilution). a) Whole muscle cell, showing UNC-38 localised to the cell membrane over the entire cell surface. Arrow A indicates the muscle cell arm, B the bag region of the cell, and C the contractile region. b) UNC-38 localised to the muscle arms (indicated by arrows) where contact with the nerve cord is made to form the neuromuscular junction. No labelling of the nerve cord itself was observed.

a)



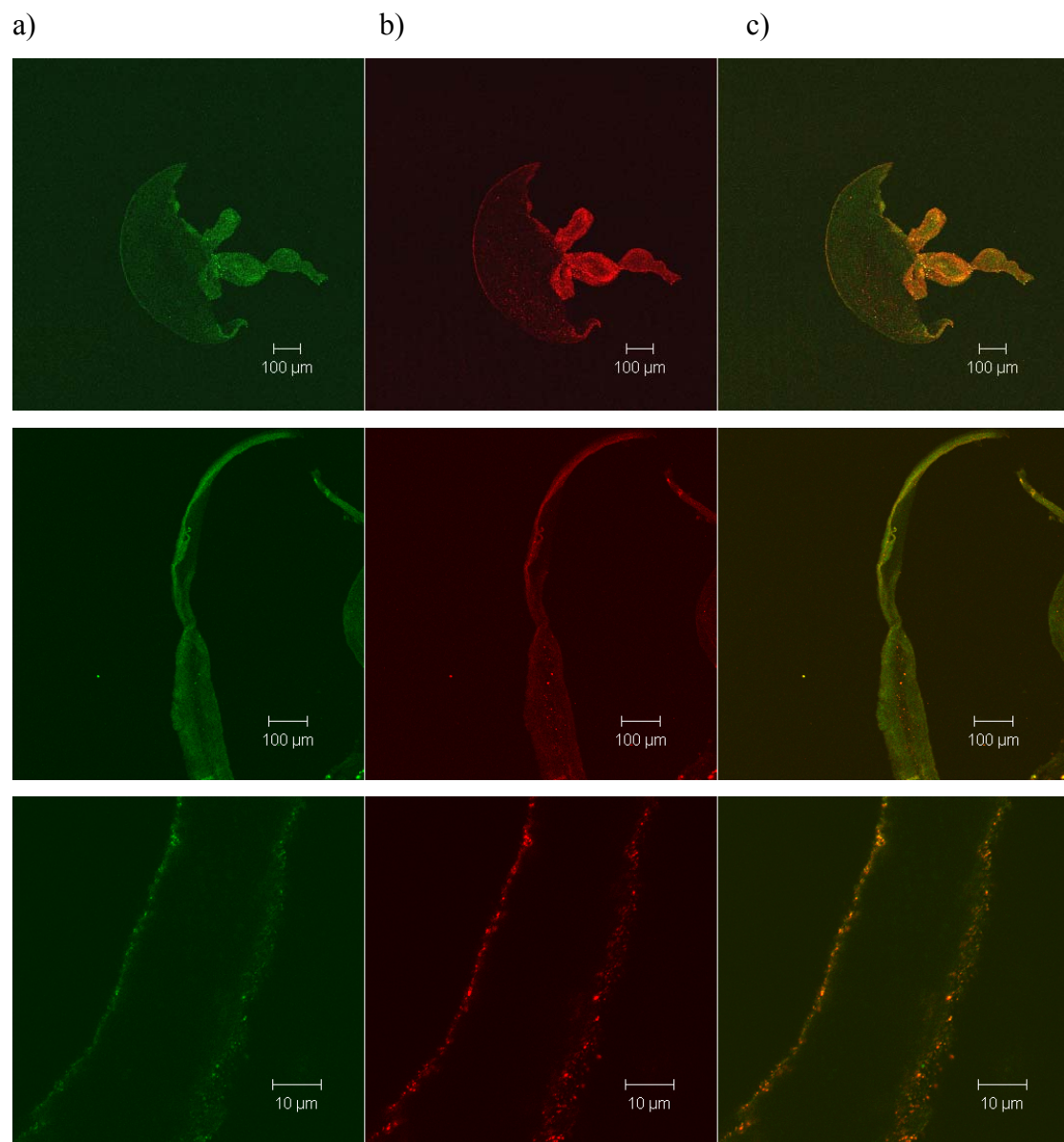
b)



**Figure 5.6: Co-localisation of *Ascaris suum* UNC-38 and UNC-29 on muscle cell membranes**

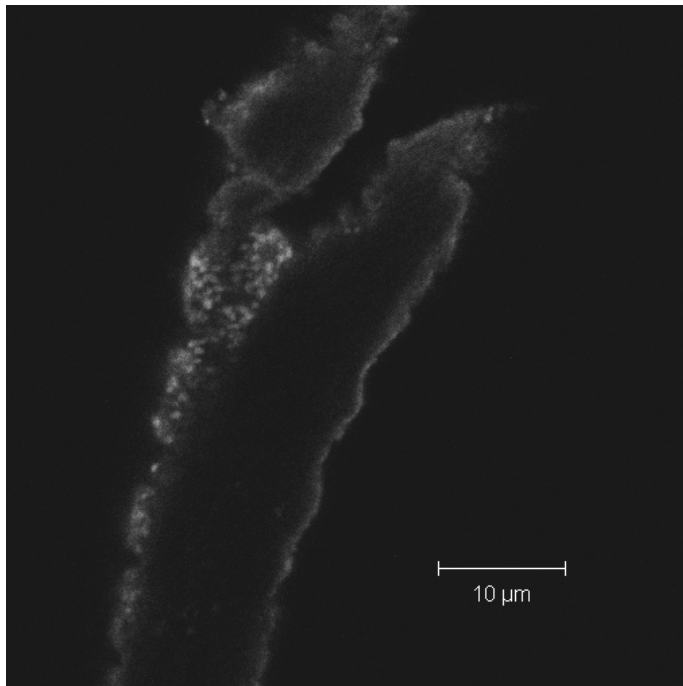
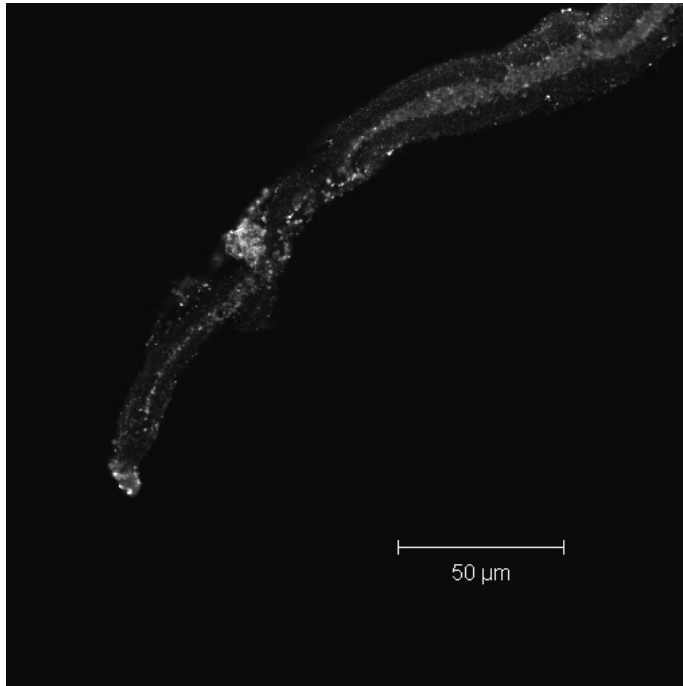
UNC-38 and UNC-29 are both localised to the *Ascaris suum* muscle cell membrane, and can be seen to co-localise, particularly on the muscle cell arm. This figure contains three sets of images showing this at progressive levels of magnification.

a) left panel: UNC-38 primary antibody (affinity purified from goat serum, 1:100 dilution) and FITC (green) conjugated secondary antibody (rabbit anti-goat, 1:200 dilution). b) central panel: UNC-29 primary antibody (affinity purified from rabbit serum, 1:100 dilution) and alexafluor 594 (red) conjugated secondary antibody (goat anti-rabbit, 1:200 dilution). c) right panel: co-localisation of UNC-38 and UNC-29 (yellow).



**Figure 5.7: Indirect immunofluorescent labelling of *Ascaris suum* UNC-63**

Indirect immunofluorescent labelling of the *Ascaris suum* muscle cell arm using primary antibody against UNC-63 (affinity purified from goat serum, 1:100 dilution) and FITC conjugated secondary antibody (rabbit anti-goat, 1:200 dilution). A greyscale image was obtained, and is shown at two different magnifications.



## **5.4 Discussion**

The strategy of raising antisera against short N-terminal subunit-specific peptides from the nAChR subunits UNC-38, UNC-29 and UNC-63 appears to have been successful; the ELISA results confirm that in all cases, immunisation resulted in the production of antibodies directed against the peptides. The negative controls carried out alongside the immunofluorescent labelling did not show any fluorescent labelling in the absence of specific primary antibody, and non-specific IgG did not bind to the *Ascaris suum* muscle cells. However, the absolute specificity of these antibodies for the target nAChR subunit can not be taken for granted; in the case of mammalian nAChRs it has been demonstrated that antibodies against  $\alpha 7$  also recognise other proteins, and will label tissue from the  $\alpha 7^{-/-}$  mouse (Jones & Wonnacott, 2005). In the case of a parasitic nematode for which transgenic techniques are unavailable, it is very difficult to confirm antibody specificity in such a manner. The specificity of our antibodies for the target nAChR has to some extent been confirmed by our collaborators at Iowa State University, who were able to partially inhibit levamisole-induced contractions of *Ascaris suum* muscle by prior incubation with the antibodies directed against UNC-38 and UNC-29 (Alan Robertson, personal communication). No such effect was seen using the antibody directed against UNC-63, and it is interesting that this is the antibody for which we failed to achieve co-localisation with the antibody directed against UNC-29. This could suggest that UNC-63 is perhaps not a component of either the UNC-29 containing nAChR or the levamisole-receptor. However, any negative results obtained by our collaborators may also be explained by the difficulties involved in sufficiently permeabilising the *Ascaris* muscle tissue to allow antibody penetration while still retaining enough integrity that the muscle will contract (Alan Robertson, personal communication). Similarly, our failure to demonstrate co-localisation may result from some interaction between the UNC-63 and UNC-29 antibodies, or may have been due to the presence of less UNC-63 protein than UNC-29, which might have led to the stronger fluorescence from UNC-29 masking weaker fluorescence from UNC-63. Unfortunately it was not possible to obtain sufficient fresh *Ascaris suum* specimens to make repeated attempts to demonstrate co-localisation of UNC-63 and UNC-29; indeed, a shortage of fresh, live



*Ascaris* specimens limited the number of times any of the immunofluorescence experiments could be carried out to 3 replicates of the UNC-38 and UNC-29 labelling, and two replicates of the UNC-63 labelling.

The results shown here demonstrate that *Ascaris suum* UNC-38 and UNC-29 are localised to the muscle cell membrane, and clearly co-localise. This is consistent with the proposed role for UNC-38 and UNC-29 as components of the same levamisole-sensitive receptor present at the *C. elegans* neuromuscular junction (Fleming *et al.*, 1997). The distribution of receptors over the whole surface of the *Ascaris suum* muscle cell differs from the receptor distribution in *C. elegans*, where the receptors are confined to clusters at the neuromuscular junction (Qian *et al.*, 2008). However, the presence of nAChRs on the bag region of the *Ascaris suum* muscle cell is well described, and single-channel electrophysiological recordings of *Ascaris* cholinergic receptors are routinely performed using the bag region of the muscle cell (Brading & Caldwell, 1971; Pennington & Martin, 1990). It is therefore likely that the distribution of fluorescent labelling observed on the *Ascaris* muscle cell does indeed represent the distribution of actual nAChRs.

## **Chapter 6: Electrophysiology**

## **6.1 Introduction**

The oocytes of the African clawed frog *Xenopus laevis* have been used for the expression of exogenous proteins since the 1970's. Initially it was discovered that injection of mRNA encoding globins into oocytes resulted in translation of the exogenous protein (Gurdon *et al.*, 1974). Subsequently, it was demonstrated that injection of mRNA or cDNA extracted from vertebrate brain resulted in the expression of neuronal membrane proteins and receptors (Ballivet *et al.*, 1988; Tomaselli *et al.*, 1989). Of particular relevance to the work presented here, it was shown that injection of *Torpedo* electric organ mRNA into *Xenopus* oocytes resulted in the production of functional nAChRs (Sumikawa *et al.*, 1984). Other studies showed that cloning, *in vitro* transcription and oocyte injection of *Torpedo* nAChR subunits also produced functional recombinant nAChRs (Mishina *et al.*, 1984). The *Xenopus* oocyte system therefore has a long established history as a useful tool for heterologous expression of ligand-gated ion channels.

The most widely used electrophysiological technique employed to study exogenous receptors and channels expressed in *Xenopus* oocytes is two-electrode voltage clamp. The principal of this technique is to control the cell membrane potential in order to allow measurements of current to be made; initially this procedure was developed to study the giant axons of squid (Hodgkin *et al.*, 1952; Marmont, 1949). The large size of the *Xenopus* oocyte makes it particularly amenable to two-electrode voltage-clamp experiments. A voltage electrode inserted through the cell membrane holds the membrane potential constant, while a second intracellular electrode applies the necessary current to maintain this membrane potential. The current is applied and the holding potential maintained by a feedback amplifier connected to the electrodes (summarised in Hille 2001). The changes in current needed to maintain the membrane potential correspond to changes in ion flow through the membrane, which occur when the ion channels under investigation open. These changes in current are converted to a digital signal and recorded by a computer equipped with appropriate software.

The *Xenopus* oocyte expression system and the two-electrode voltage clamp technique have been used to study ligand-gated ion channels of both vertebrates and invertebrates. The system allows the proposed function of cloned cDNAs showing sequence homology with other LGICs to be confirmed (Schofield *et al.*, 1987).

The LGICs of the model organisms *C. elegans* and *Drosophila melanogaster* have been extensively studied by expression in *Xenopus* oocytes (Bianchi & Driscoll, 2006; Buckingham *et al.*, 2006). The glutamate-gated chloride channels of *C. elegans* have been expressed in *Xenopus* oocytes and used to confirm the mode of action of the avermectin anthelmintics (Arena *et al.*, 1992). The *Drosophila* GABA receptor RDL expresses most efficiently and is a useful screen for the ability of potential insecticide resistance mutations to alter the pharmacology of the receptor (French-Constant & Rocheleau, 1993; Le Goff *et al.*, 2005).

The nicotinic acetylcholine receptors of invertebrates have also been expressed in *Xenopus* oocytes for two-electrode voltage clamp experiments, though perhaps with less success than the ligand-gated chloride channels. Typically, currents induced by agonist application are much smaller, though this can in some cases be improved by co-expression of a relevant accessory protein (Halevi *et al.*, 2002).

In the case of heteromeric nAChRs, expression can be impaired by the lack of appropriate complementary subunits, though this can sometimes be overcome by the construction of chimaeras or the co-expression of subunits from a different species (Ihara *et al.*, 2003; Shimomura *et al.*, 2005). Despite these potential difficulties, the *Xenopus* oocyte expression system remains a useful tool for expressing invertebrate nicotinic acetylcholine receptors for two-electrode voltage clamp. An example of particular relevance to the work presented here is the successful expression of proposed components of the *C. elegans* levamisole receptor UNC-38, UNC-29 and LEV-1, which formed a functional levamisole-sensitive nAChR (Fleming *et al.*, 1997).

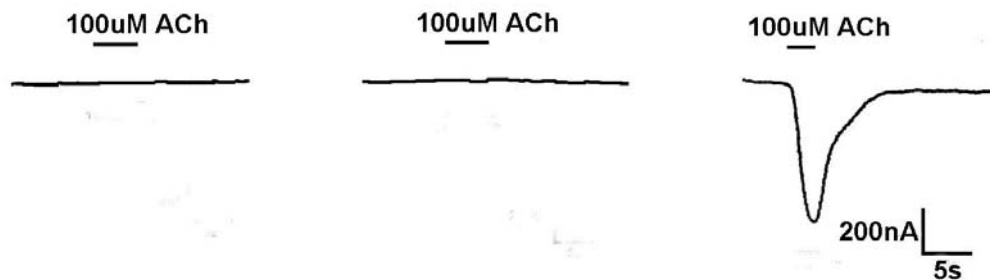
## **6.2: Initial experiments to co-express *Ascaris unc-38* and *unc-29***

Preliminary experiments were performed to demonstrate that *Ascaris unc-38* and *unc-29* did indeed co-express to form a heteromeric receptor. Oocytes were injected either with 25ng of *Ascaris unc-38* cRNA, 25ng of *Ascaris unc-29* cRNA, or 25ng *Ascaris unc-38*+25ng *Ascaris unc-29* cRNA. All injection volumes were adjusted to 50nl using nuclease-free water. The results of this are shown in Figure 6.1, and clearly demonstrate that both *Ascaris unc-38* and *unc-29* are required to produce a functional nAChR which responds to ACh. The response to 100μM ACh was ~600-800nA, varying between different oocytes.

Preliminary pharmacological experiments were then performed to discover which agonists the heterologously expressed *Ascaris* nAChR responded to, and to ensure that such responses could be blocked with an appropriate antagonist to confirm a genuine nAChR response. The agonists used were nicotine, ACh and levamisole; it has been demonstrated that in *C. elegans*, UNC-38 and UNC-29 are components of the levamisole-sensitive nAChR (Fleming *et al.*, 1997). The antagonist chosen was mecamylamine; this is a broad-spectrum non-competitive antagonist of nAChRs, and has additionally been shown to inhibit ACh-induced contractions and currents in native *Ascaris* muscle preparations (Colquhoun *et al.*, 1991; Natoff, 1969). The results are shown in Figure 6.2. The receptors composed of *Ascaris unc-38* and *unc-29* clearly respond to 100μM concentrations of acetylcholine, nicotine and levamisole; the response to levamisole appeared slightly larger than the response to ACh, which was in turn slightly larger than the response to nicotine. The responses to all these agonists can be fully blocked by application of 10μM mecamylamine, and the response recovers when the mecamylamine is washed off. The response to nicotine, and the blockade by mecamylamine, confirm that the ACh response is nicotinic in nature, rather than muscarinic. The relatively large response to levamisole confirms that in *Ascaris suum*, UNC-38 and UNC-29 are components of a levamisole-sensitive nAChR, as they are in *C. elegans*.

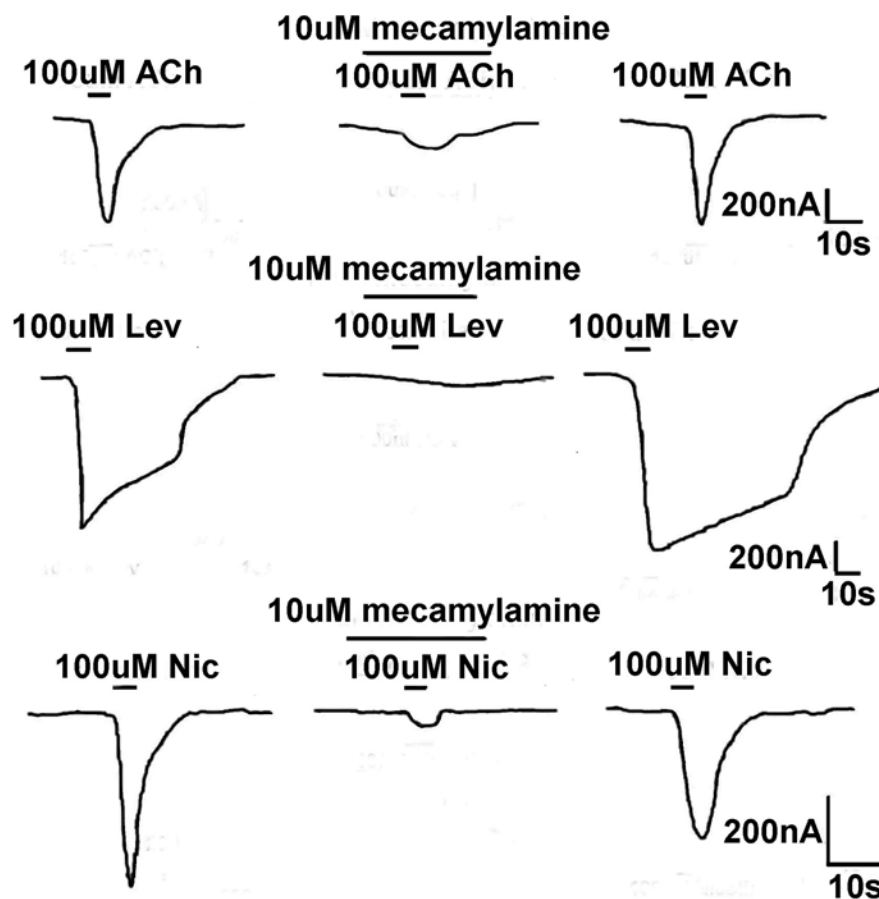
**Figure 6.1: Both *Ascaris unc-38* and *unc-29* are required to form a functional nAChR**

Both UNC-38 and UNC-29 are required to produce a functional nAChR. Left-hand trace: there is no response to application of ACh from an oocyte injected with *unc-38* RNA alone. Centre trace: There is no response to application of ACh from an oocyte injected with *unc-29* RNA alone. Right-hand trace: Application of 100 $\mu$ M ACh to an oocyte injected with an equimolar ratio of *unc-38* and *unc-29* RNA produces a ~600-800nA response.



**Figure 6.2: The nAChRs composed of UNC-38 and UNC-29 are sensitive to the agonists ACh, levamisole and nicotine, and can be reversibly blocked by the antagonist mecamylamine.**

Left panels: co-expression of *unc-38* and *unc-29* produces nAChRs which respond to application of 100 $\mu$ M acetylcholine (top), levamisole (centre) and nicotine (bottom). Centre panels: All these responses can be blocked by application of 10 $\mu$ M mecamylamine. Right panels: All responses recover after the antagonist has been washed off.

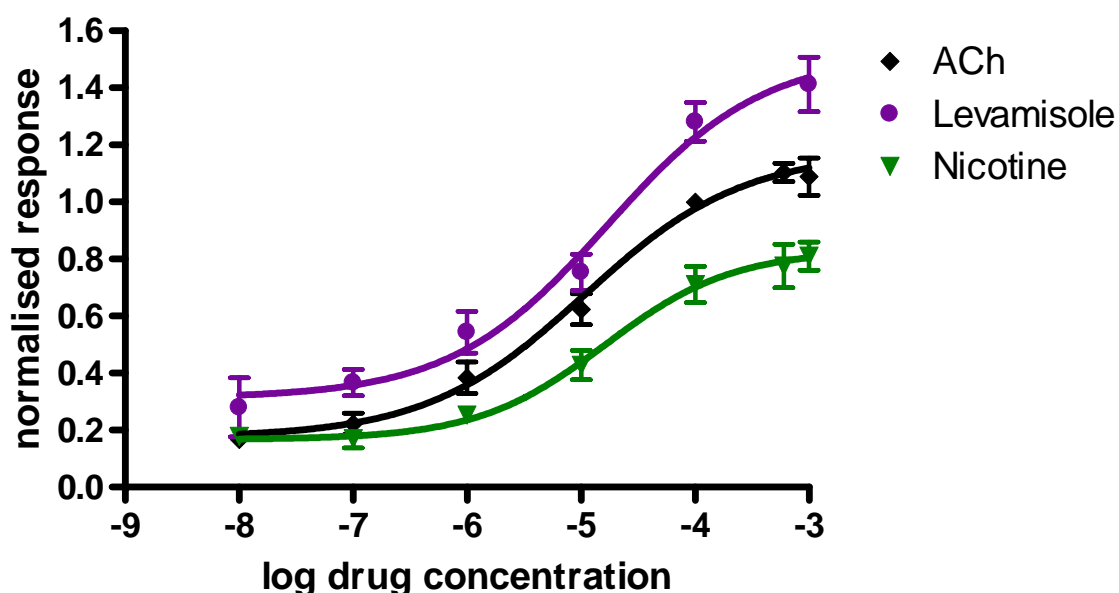


Dose-response experiments were carried out using oocytes injected with equimolar ratios of *Ascaris unc-38* and *unc-29*, and the agonists levamisole, ACh, and nicotine. The recorded responses from each oocyte were normalised to the response to 100 $\mu$ M ACh, and were analysed using GraphPad Prism software. Sigmoidal dose-response curves were fitted to each dataset, and the EC<sub>50</sub> and Hill slope coefficients obtained from the curves. These graphs are shown in Figure 6.3, and the EC<sub>50</sub> values and Hill coefficients are summarised in Table 6.1. The order of agonist efficacy appears to be levamisole>ACh>nicotine, though the EC<sub>50</sub> values suggest these compounds show similar potency. It is possible that if the data were reanalysed to measure area under the peak rather than peak height, the results may show levamisole to be more potent, as application of this drug produced broader peaks. This is consistent with observations from native *Ascaris* muscle (Colquhoun *et al.*, 1991; Harrow & Gration, 1984). However, studies of native muscle will always contain recordings from a mixed population of receptors with different pharmacological properties; the graphs presented here, with their very shallow slope and a large variation in the measurements of Hill slope, may also be indicative of a mixed receptor population. Indeed, the pentameric nature of the nAChR itself suggests that injecting equimolar concentrations of cRNA for 2 subunits is unlikely to produce a homogenous population; early experiments expressing  $\alpha 4\beta 2$  in *Xenopus* oocytes produced mixed receptor populations resulting in rather flattened dose response curves with shallow slopes (Zwart & Vijverberg, 1998). These curves were interpreted as resulting from receptors with different stoichiometries having different pharmacological properties, and prompted experiments using different ratios of  $\alpha 4:\beta 2$  RNA to influence the receptor stoichiometry (Zwart & Vijverberg, 1998). Knowledge of this work suggested that further experiments are required to investigate whether the pharmacology of recombinant *Ascaris* nAChRs is affected by stoichiometry.



**Figure 6.3: Dose-response relationships for the agonists levamisole, ACh and nicotine applied to oocytes injected with equimolar concentrations of *Ascaris unc-38* and *unc-29* cRNA**

All responses were normalised to the response of each oocyte to 100 $\mu$ M ACh, and sigmoidal dose-response curves were fitted to the data using GraphPad Prism. N=3 (where N is batches of oocytes) and n=6 (where n is number of individual oocytes) minimum for each data point. The order of agonist efficacy is levamisole > ACh > nicotine.



**Table 6.1: EC<sub>50</sub> values and Hill slope values for oocytes injected with equimolar concentrations of *Ascaris unc-38* and *unc-29* cRNA**

Agonist	ACh	Levamisole	Nicotine
EC <sub>50</sub>	1.07e-005	1.68e-005	1.62e-005
95% confidence intervals of EC <sub>50</sub>	5.19e-006 to 2.21e-005	5.12e-006 to 5.49e-005	5.84e-006 to 4.48e-005
Hill slope	0.625	0.635	0.774
95% confidence intervals of Hill slope	0.353 to 0.897	0.181 to 1.09	0.137 to 1.41

### **6.3 Experiments using oocytes injected with 1:5 and 5:1 ratios of *Ascaris unc-38* and *unc-29* cRNA**

Oocytes were injected with *Ascaris unc-38* + *unc-29* cRNA in the ratios 1:5 and 5:1 *unc-38:unc-29*. The total amount of RNA injected was kept constant at 50ng per oocyte, in an injection volume of 50nl. It was hoped that this would drive the stoichiometry of the expressed receptors to either 3:2 UNC-38:UNC-29 or 2:3 UNC-38:UNC-29, to allow any difference in pharmacology between the two types to be further investigated. Although potentially receptors with a 4:1 or 1:4 stoichiometry could also be generated, it has previously been demonstrated by transfection of cells with  $\alpha 4$  and  $\beta 2$  cDNA that the stoichiometry of such heteromeric receptors composed of just 2 subunits is 3:2 or 2:3, and that adjusting the subunit cDNA ratios drove the receptors to one stoichiometry or the other without introducing new combinations (Nelson *et al.*, 2003). Preliminary experiments suggested that the 1:5 and 5:1 RNA ratios were optimal for this experiment; although studies of the human  $\alpha 4\beta 2$  receptor have used 10:1 or 9:1 ratios to drive changes in receptor stoichiometry (Moroni *et al.*, 2006; Nelson *et al.*, 2003; Zwart & Vijverberg, 1998). 1:10 and 10:1 ratios of *Ascaris unc-38:unc-29* RNA either did not produce functional receptors, or resulted in very low expression levels where the maximal current was <20nA.

Dose-response experiments using the agonists levamisole and nicotine were carried out on the receptors expressed from injection of the different RNA ratios. Maximal responses from oocytes injected with the 1:5 ratio were ~300-400nA; for the 5:1 ratio, ~100-200nA. The responses from each oocyte were normalised to the response to 100 $\mu$ M ACh, and were again analysed using GraphPad Prism as described in Results section 1. Dose response relationships and examples of electrophysiology traces for oocytes injected with a 1:5 ratio of *unc-38:unc-29* RNA are shown in Figure 6.4. Dose response relationships and examples of electrophysiology traces for oocytes injected with a 5:1 ratio of *unc-38:unc-29* RNA are shown in Figure 6.5. The EC<sub>50</sub> and Hill slope values are presented in Table 6.2.

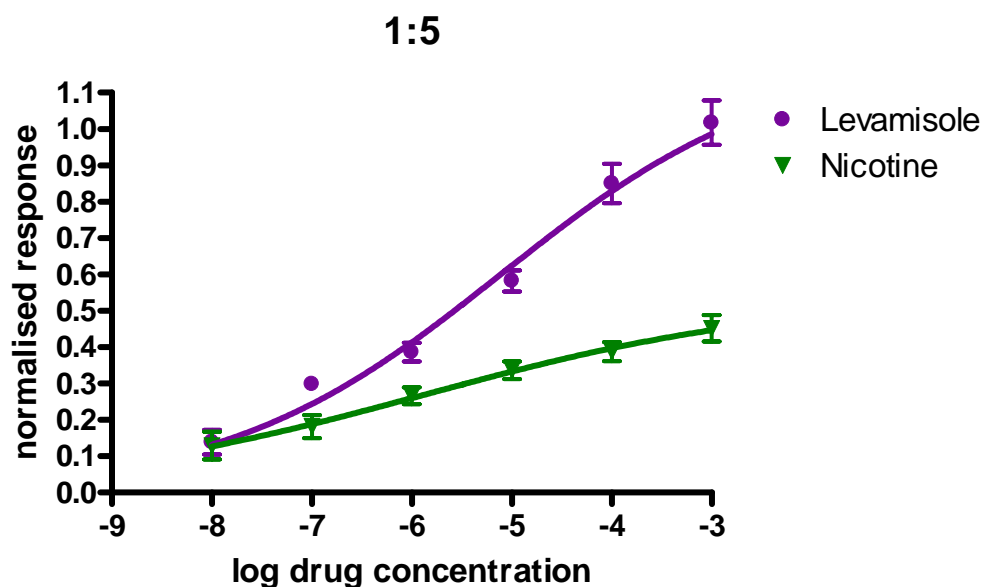
Changing the stoichiometry of the *Ascaris* nAChR by using different RNA ratios clearly has an effect on the pharmacology. The 1:5 ratio produces a receptor population which is more sensitive to levamisole, whereas nicotine is only a partial agonist. In contrast to this, the 5:1 ratio produces a receptor population which is most sensitive to nicotine, whereas levamisole is only a partial agonist. It cannot be inferred

from the data collected here whether the partial activity of the less efficacious compound resulted from a true partial agonism of an almost homogeneous receptor population, or from full agonist activity at a sub-population of receptors with the opposite stoichiometry to that of the majority in a heterogeneous population. Once again, the relatively shallow slope of the graphs may suggest a mixed receptor population, but attempts to fit two separate curves to each dataset using Graphpad Prism were unsuccessful.

**Figure 6.4: Dose-response relationships for levamisole and nicotine for oocytes injected with a 1:5 ratio of *unc-38:unc-29* cRNA**

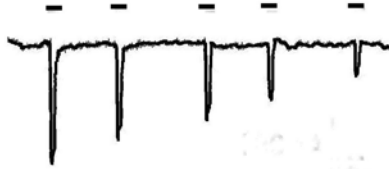
A 1:5 ratio of *unc-38:unc-29* RNA produces a receptor population most sensitive to levamisole and less sensitive to nicotine. a) Dose-response relationships for levamisole and nicotine from oocytes injected with a 1:5 ratio of *unc-38:unc-29*. All data was normalised to the response to 100 $\mu$ M ACh for each individual oocyte. N=3 n=6 minimum for each data point, where N is the number of batches of oocytes, and n is the number of individual oocytes. Sigmoidal dose-response curves were fitted using GraphPad Prism. b) Example electrophysiology traces from an oocyte injected with a 1:5 ratio of *unc-38:unc-29* in response to the application of levamisole (upper trace) and nicotine (lower trace). The currents evoked by levamisole application are larger than those produced by nicotine application. Agonist application is indicated by a bar above the trace. Agonist concentrations were applied in the order 1mM, 100 $\mu$ M, 10 $\mu$ M, 1 $\mu$ M then 100nM.

a)

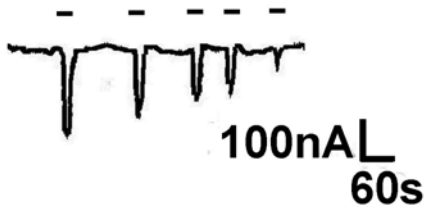


b)

**levamisole**



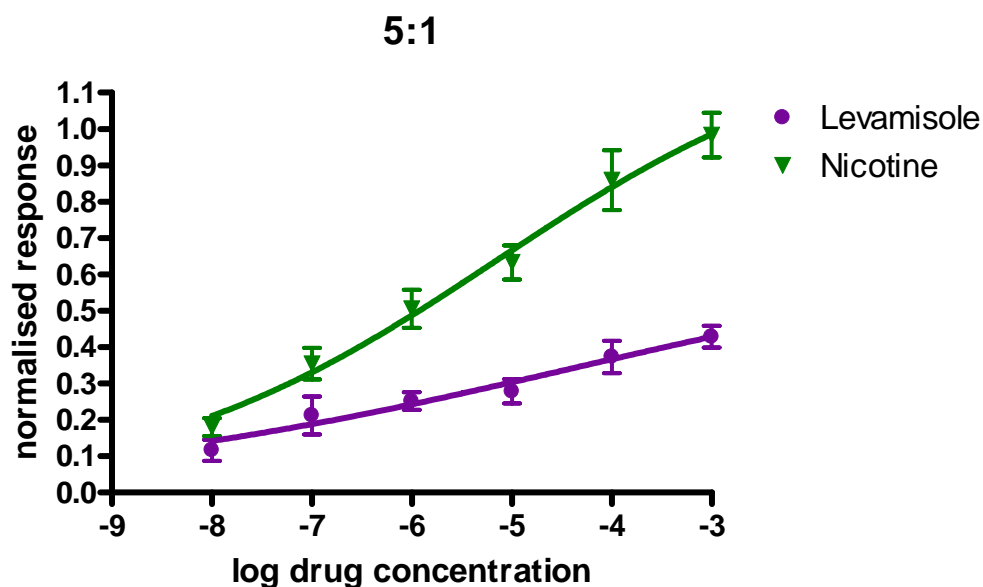
**nicotine**



**Figure 6.5: Dose-response relationships for levamisole and nicotine for oocytes injected with a 5:1 ratio of *unc-38:unc-29* cRNA**

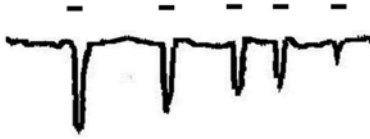
A 5:1 ratio of *unc-38:unc-29* RNA produces a receptor population most sensitive to nicotine and less sensitive to levamisole. a) Dose-response relationships for levamisole and nicotine from oocytes injected with a 5:1 ratio of *unc-38:unc-29*. All data was normalised to the response to 100 $\mu$ M ACh for each individual oocyte. N=3 n=6 minimum for each data point. Sigmoidal dose-response curves were fitted using GraphPad Prism. b) Example electrophysiology traces from an oocyte injected with a 5:1 ratio of *unc-38:unc-29* in response to the application of levamisole (upper trace) and nicotine (lower trace). The currents evoked by levamisole application are larger than those produced by nicotine application. Agonist application is indicated by a bar above the trace. Agonist concentrations were applied in the order 1mM, 100 $\mu$ M, 10 $\mu$ M, 1 $\mu$ M then 100nM.

a)

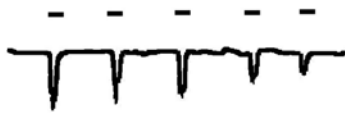


b)

**nicotine**



**levamisole**



200nA   
60s

**Table 6.2: EC<sub>50</sub> and Hill slope values for oocytes injected with 1:5 and 5:1 ratios of *Ascaris unc-38:unc-29* RNA**

RNA Ratio ( <i>unc-38:unc-29</i> )	1:5	1:5	5:1	5:1
Agonist	Levamisole	Nicotine	Levamisole	Nicotine
EC <sub>50</sub>	7.74e-006	1.33e-006	4.15e-005	6.93e-006
95% confidence interval EC <sub>50</sub>	9.53e-007 to 6.29e-005	1.65e-008 to 1.06e-004	7.85e-015 to 219000	8.49e-008 to 5.66e-004
Hill slope	0.314	0.240	0.161	0.247
95% confidence interval of Hill slope	0.201 to 0.427	0.0673 to 0.412	-0.0672 to 0.389	0.107 to 0.388

Although changing the receptor pharmacology by adjusting the stoichiometry is well described for the mammalian  $\alpha 4\beta 2$  receptor (Moroni & Bermudez, 2006; Moroni *et al.*, 2006; Nelson *et al.*, 2003), this has not previously been demonstrated in invertebrates. The striking difference in sensitivity to levamisole and nicotine observed between receptor populations driven to different stoichiometry also raised the possibility that two of the observed pharmacological subtypes found in native *Ascaris* muscle (the L- and N- subtypes) could in fact be generated using just these two nAChR subunits, UNC-38 and UNC-29 (Levandoski *et al.*, 2005). It was therefore necessary to test this hypothesis using different agonist compounds known to be specific for these different pharmacological nAChR subtypes.

#### **6.4 Further pharmacological studies on oocytes injected with 1:5 and 5:1 ratios of *Ascaris unc-38* and *unc29* cRNA**

Oocytes were again injected with 1:5 or 5:1 ratios of *Ascaris unc-38:unc-29* cRNA, to produce receptor populations with predominantly 2:3 or 3:2 UNC-38:UNC-29 stoichiometry. Of the available agonist compounds, pyrantel and oxantel were chosen for comparative pharmacology because in addition to their respective specificity for the L- and N-subtypes (Martin *et al.*, 2004; Qian *et al.*, 2006; Robertson *et al.*, 1994) they are anthelmintic drugs of medical and veterinary relevance. Dose-response experiments were performed on the oocytes injected with 1:5 and 5:1 ratios of *Ascaris unc-38:unc-29* cRNA using pyrantel and oxantel. The action of these compounds is more complex than the simple agonist properties of nicotine, levamisole and ACh; pyrantel and oxantel are known to be agonists of the native *Ascaris* nAChRs at low concentrations, but cause open channel block at higher concentrations, producing an asymmetric bell-shaped dose-response relationship (Dale & Martin, 1995; Harrow & Gration, 1984). The oocytes injected with a 1:5 ratio of *Ascaris unc-38:unc29* cRNA were much more sensitive to pyrantel, showing very little response to the application of oxantel. The responses for this experiment were all normalised to the maximal response to pyrantel, which occurred at a concentration of 1 $\mu$ M. Dose-response relationships for pyrantel and oxantel from oocytes injected with a 1:5 ratio of *Ascaris unc-38:unc-29* cRNA are shown in Figure 6.6a; examples of the electrophysiology traces obtained from these experiments are shown in Figure 6.6b. The oocytes



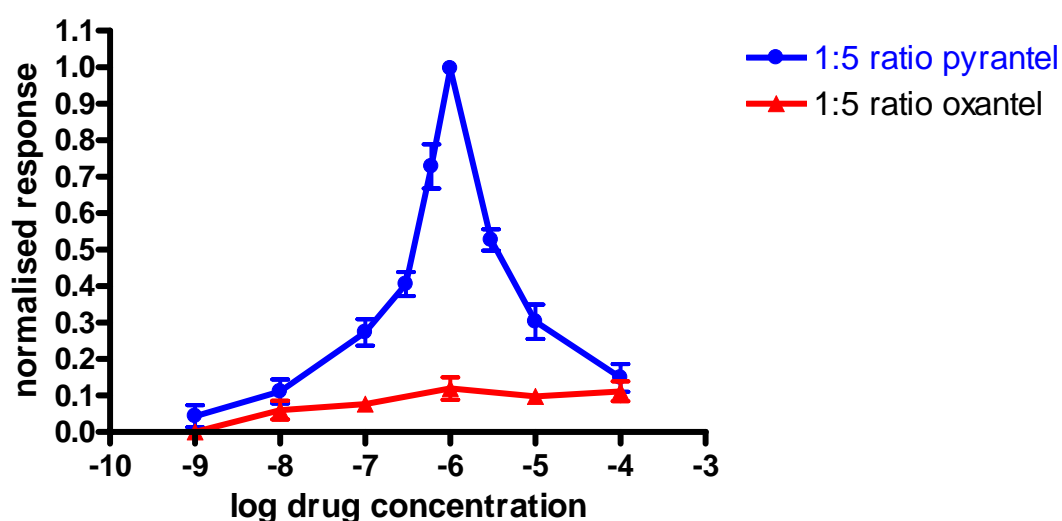
injected with a 5:1 ratio of *Ascaris unc-38:unc-29* cRNA were much more sensitive to oxantel, showing very little response to the application of pyrantel. The responses for this experiment were therefore all normalised to the maximal response to oxantel, which occurred at a concentration of 10 $\mu$ M. Dose-response relationships for pyrantel and oxantel from oocytes injected with a 1:5 ratio of *Ascaris unc-38:unc-29* cRNA are shown in Figure 6.7a; examples of the electrophysiology traces obtained from these experiments are shown in Figure 6.7b. The data were initially plotted simply as points on graphs of the logarithm of drug concentration against the normalised response; attempts to utilise an appropriate equation to fit a curve with two components to the data (Moroni *et al.*, 2008; Whiteaker *et al.*, 1998) were unsuccessful, probably due to the steepness of the slopes. In order to obtain estimates of the EC<sub>50</sub> and IC<sub>50</sub> values for the actions of pyrantel and oxantel on the expressed *Ascaris* nAChRs, the data was split into agonist and antagonist components, which were analysed separately in Graphpad Prism as described in section 6.2. These graphs are shown in Figure 6.8 and the EC<sub>50</sub>, IC<sub>50</sub> and Hill slope values are summarised in Table 6.3.

The steepness of the graphs, which did not flatten as they approached I<sub>max</sub>, caused sigmoidal dose-response relationships to be a poor fit for the data. It is likely that if the agonist and antagonist components did not intersect, then the top of the graphs would exceed the observed I<sub>max</sub>. This would mean that the potential maximal current would greatly exceed the maximal response to acetylcholine; this could perhaps be explained by the described action of oxantel as an allosteric modulator (Raymond *et al.*, 2000), or one could speculate that pyrantel and oxantel act as super-agonists, in a similar manner to the neonicotinoid insecticide clothianidin (Ihara *et al.*, 2004). Unfortunately it was beyond the scope of this particular experiment to further separate the agonist and antagonist components of the responses to pyrantel and oxantel. However, the data clearly shows that oocytes injected with a 1:5 ratio of *Ascaris unc-38:unc-29* cRNA are much more sensitive to pyrantel, while the 5:1 ratio produces receptors much more sensitive to oxantel. This supports the hypothesis that the 1:5 ratio may induce expression of the L-type nAChR observed in *Ascaris suum* muscle, most sensitive to levamisole and pyrantel; while the 5:1 ratio may induce expression of the N-subtype nAChR, most sensitive to nicotine and oxantel.

**Figure 6.6: Dose-response relationships for pyrantel and oxantel applied to oocytes injected with a 1:5 ratio of *Ascaris unc-38:unc-29* cRNA.**

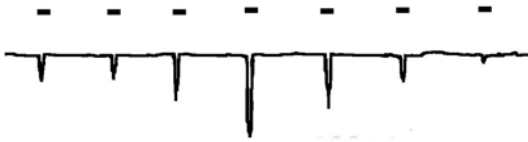
A 1:5 ratio of *unc-38:unc-29* RNA produces a receptor population most sensitive to pyrantel and much less sensitive to oxantel. a) Dose-response relationships for pyrantel and oxantel applied to oocytes injected with a 1:5 ratio of *Ascaris unc-38:unc-29*. All data was normalised to the response to 1 $\mu$ M pyrantel for each individual oocyte. N=3 n=6 minimum for each data point. b) Example electrophysiology traces from an oocyte injected with a 1:5 ratio of *unc-38: unc-29* in response to the application of pyrantel (upper trace) and oxantel (lower trace). The currents evoked by pyrantel application were much larger than those produced by oxantel application. Agonist application is indicated by a bar above the trace. Pyrantel concentrations were applied in the order 100 $\mu$ M, 10 $\mu$ M, 3 $\mu$ M, 1 $\mu$ M, 600nM, 100nM, 10nM. Oxantel concentrations were applied in the order 100 $\mu$ M, 10 $\mu$ M, 1 $\mu$ M, 100nM, 10nM.

a)

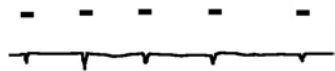



b)

**pyrantel**



**oxantel**

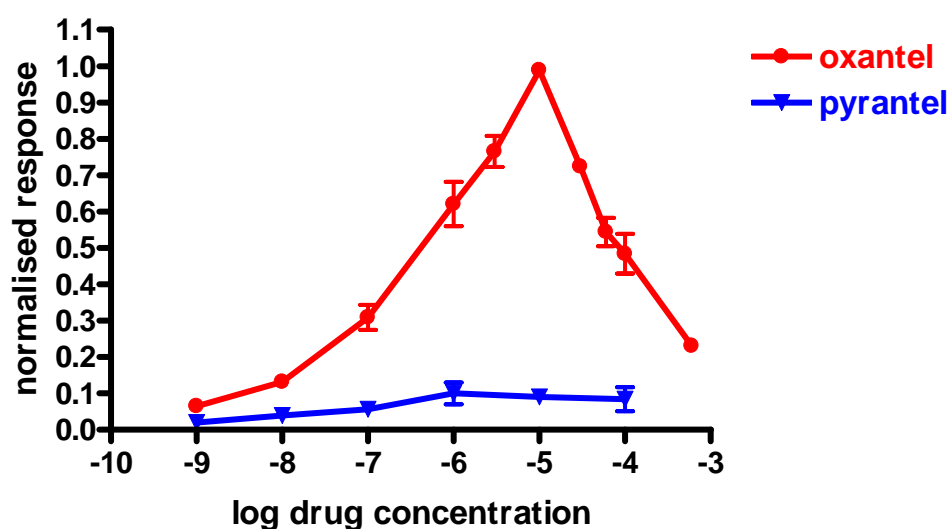


200nA   
50s

**Figure 6.7: Dose-response relationship for oxantel and pyrantel applied to oocytes injected with a 5:1 ratio of *Ascaris unc-38:unc-29* cRNA.**

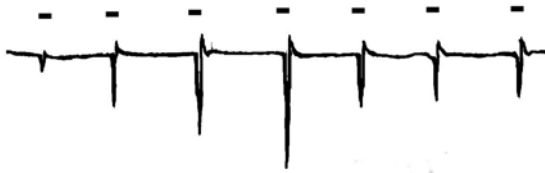
A 5:1 ratio of *Ascaris unc-38:unc-29* cRNA produces a receptor population most sensitive to oxantel and much less sensitive to pyrantel. a) Dose-response relationships for pyrantel and oxantel applied to oocytes injected with a 5:1 ratio of *Ascaris unc-38:unc-29*. All data was normalised to the response to 10 $\mu$ M oxantel for each individual oocyte. N=3 n=6 minimum for each data point. b) Example electrophysiology traces from an oocyte injected with a 5:1 ratio of *unc-38: unc-29* in response to the application of oxantel (upper trace) and pyrantel (lower trace). The currents evoked by oxantel application were much larger than those produced by pyrantel application. Agonist application is indicated by a bar above the trace. Oxantel concentrations were applied in the order 100nM, 1 $\mu$ M, 6 $\mu$ M, 10 $\mu$ M, 60 $\mu$ M, 100 $\mu$ M, 600 $\mu$ M. Pyrantel concentrations were applied in the order 100 $\mu$ M, 10 $\mu$ M, 1 $\mu$ M, 100nM, 10nM.

a)

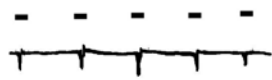



b)

**oxantel**



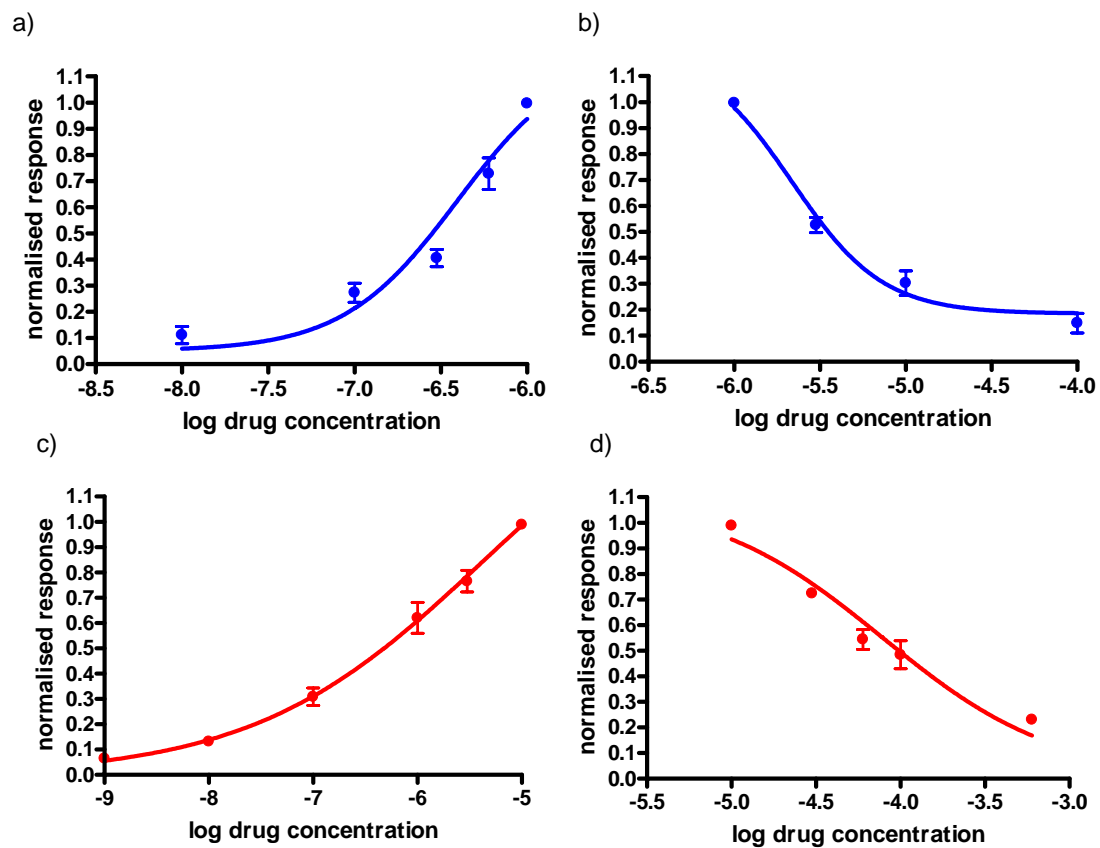
**pyrantel**



200nA   
50s

**Figure 6.8: Sigmoidal curves fitted to separate agonist and antagonist components of dose-response data for pyrantel and oxantel**

GraphPad Prism was used to fit sigmoidal dose response curves to the separate agonist and antagonist components of the normalised dose-response data for pyrantel on oocytes injected with the 1:5 ratio of *Ascaris unc-38:unc-29* cRNA (panels a and b) and for oxantel on oocytes injected with a 5:1 ratio of *Ascaris unc-38:unc-29* cRNA (panels c and d).



**Table 6.3: EC<sub>50</sub>, IC<sub>50</sub> and Hill slope values for oxantel and pyrantel on oocytes injected with a 1:5 and 5:1 ratio of *Ascaris unc-38:unc-29* RNA**

Data obtained from fitting separate dose-response curves to agonist and antagonist components of normalised dose-response data using GraphPad Prism. Values are shown to 3 significant figures.

RNA ratio ( <i>unc-38:unc-29</i> )	1:5	5:1
Compound	Pyrantel	Oxantel
EC <sub>50</sub>	3.95e-007	4.06e-006
95% confidence interval EC <sub>50</sub>	1.73e-007 to 9.01e-007	3.03e-008 to 5.43e-004
Hill slope (agonist component)	1.31	0.397
95% confidence interval Hill slope	0.484 to 2.14	-0.00191 to 0.794
IC <sub>50</sub>	2.17e-006	7.86e-005
95% confidence interval IC <sub>50</sub>	9.55e-007 to 4.95e-006	4.06e-005 to 1.52e-004
Hill slope (antagonist component)	-1.64	-0.843
95% confidence interval Hill slope	-2.99 to -0.285	-1.19 to -0.494

## **6.5 Discussion**

The heterologous expression of functional levamisole-sensitive nAChRs using the *Ascaris suum* homologues of *unc-38* and *unc-29* confirms that in *Ascaris*, as in *C. elegans*, these subunits are likely to be components of the native levamisole-sensitive nAChR (Fleming *et al.*, 1997). However, in *C. elegans*, it has been reported that 3 other nAChR subunits are necessary for the levamisole response: *unc-63*, *lev-1*, and *lev-8* (Culetto *et al.*, 2004; Fleming *et al.*, 1997; Towers *et al.*, 2005). We have shown using a bioinformatics approach and the genome of *Brugia malayi* and a parasite closely related to *Ascaris suum* that *lev-1* and *lev-8* are apparently absent in this group of nematodes; however *unc-63* is apparently present and well-conserved. The functional significance of *unc-63* in *Ascaris* is currently unknown, and further experiments, where *Ascaris unc-63* would be co-expressed with *unc-38* and *unc-29*, are necessary to elucidate what effect this subunit would have on receptor pharmacology. It is possible that the third pharmacological nAChR subtype described from *Ascaris*, the B-subtype, most sensitive to buphenium, may contain the UNC-63 subunit (Qian *et al.*, 2006; Robertson *et al.*, 2002).

If the pharmacology of *Ascaris* nAChRs can be altered simply by generating different combinations of two subunits, this may help to explain how sufficient pharmacological and neurological complexity can be found in parasites with remarkably few nAChR genes compared to the model nematode *C. elegans*. Pharmacological diversity generated by alternate stoichiometries have not previously been described from invertebrates, but are well described for heterologous expression of the mammalian neuronal nAChR subunits  $\alpha 4$  and  $\beta 2$  (Nelson *et al.*, 2003; Zwart & Vijverberg, 1998). Receptors with the stoichiometry  $(\alpha 4)_3(\beta 2)_2$  have lower agonist affinity and a different pharmacological profile to receptors with the stoichiometry  $(\alpha 4)_2(\beta 2)_3$ , and addition of an accessory subunit to give (for example) an  $(\alpha 4)_2(\beta 2)_2(\alpha 5)$  combination confers additional pharmacological differences (Kuryatov *et al.*, 2008; Moroni *et al.*, 2006). In extrapolating the  $\alpha 4\beta 2$  model to interpret the results presented here, we may speculate that in *Ascaris* the 3 pharmacological subtypes may have the stoichiometry  $(UNC-38)_2(UNC-29)_3$ ,  $(UNC-38)_3(UNC-29)_2$ , and  $(UNC-38)_2(UNC-29)_2(UNC-63)$ , where  $(UNC-38)_2(UNC-29)_3$  constitutes the L-



subtype, (UNC-38)<sub>3</sub>(UNC-29)<sub>2</sub> forms the N-subtype, and addition of UNC-63 as an accessory subunit may potentially create the B-subtype. Further work is necessary to confirm or refute this hypothesis: the stoichiometry of the receptors expressed here is only inferred, and experiments where receptor stoichiometry is more clearly defined would use concatamers composed of *Ascaris unc-38* and *unc-29* cRNAs joined together, to which a small quantity of accessory subunit cRNA (*Ascaris unc-38*, *unc-29* or *unc-63*) would be added. Patch-clamp experiments to make single channel recordings of the recombinant *Ascaris* nAChRs would also be very informative, as the native *Ascaris* nAChRs have been characterised on a single-channel level (Levandoski *et al.*, 2005). In addition to this, the pharmacology of the heterologously expressed putative L- and N-type nAChRs could be further tested using other agonist and antagonist compounds; methyridine is an additional agonist which should activate the N-subtype, while paraherquamide is an antagonist specific to the L-subtype (Martin *et al.*, 2004; Robertson *et al.*, 2002). Unfortunately it was not possible to obtain these compounds during the experiments presented here.

The physiological relevance of different pharmacological subtypes of nAChR in the same tissue is unknown, but it is possible that if the receptors possess different affinities for the native agonist ACh, then the more sensitive receptors may be primarily extrasynaptic, while the less sensitive subtype may be more likely to be found clustered at the synapse; this is suggested as the possible physiological relevance of mammalian receptors with different agonist affinities by Nelson *et al.* (2003). However, as all single channel measurements of native *Ascaris* nAChRs have to date been from pools of extrasynaptic receptors, it is not known whether this interpretation is relevant to the properties of nematode nAChRs.

In summary, we have demonstrated that the *Ascaris* homologues of *unc-38* and *unc-29* co-express in *Xenopus* oocytes to form a functional nAChR, which is the first ever successful expression of a parasite nAChR. We have also shown that changing the receptor stoichiometry also changes the pharmacology, which has not previously been described for invertebrate nAChRs. The nAChRs reconstituted here show similar pharmacological profiles to the native *Ascaris* nAChRs described as the N-subtype (most sensitive to nicotine and oxantel) and the L-subtype (most sensitive to levamisole and pyrantel). The results presented here have important implications for the development and management of parasite resistance to cholinergic anthelmintics; it has previously been assumed that parasites resistant to levamisole or pyrantel may

still be susceptible to oxantel, as different receptor sub-types are involved (Martin *et al.*, 2004). If the target receptors of all these cholinergic anthelmintics contain the same subunits, a point mutation in one of the subunits which affects drug binding will be present in two distinct drug targets, increasing the likelihood of cross-resistance. If a more serious loss of function mutation were to occur under anthelmintic selection, the converse argument could be made: that cross-resistance is unlikely, as loss of one functional subunit may result in the loss of 2 or 3 different receptor subtypes and seriously affect cholinergic signalling in the nematode. However, McCarthy and colleagues have demonstrated that in hookworms, *unc-38*, *unc-29* and *unc-63* are all expressed at lower levels in pyrantel resistant populations, and this may be partially compensated for by the upregulation of another nAChR subunit, *acr-19* (Kopp *et al.*, 2008). If a similar resistance mechanism was to occur in *Ascaris suum*, and an uncharacterised subunit could take over the role of cholinergic signalling at the neuromuscular junction, then cross-resistance to levamisole, pyrantel and oxantel may still become a problem.

## **Chapter 7: Conclusions**

The aim of the work presented here was to interpret the described pharmacology of the native neuromuscular nAChRs of *Ascaris suum* in terms of the underlying molecular biology, in particular the levamisole-sensitive nAChR which is the target of the cholinergic anthelmintic drugs levamisole and pyrantel. Prior to the start of this work, such investigations had previously been confined to the model nematode *C.elegans*, though during the course of the work presented here more recent studies emerged associating altered expression levels of nAChR subunits with drug resistance in the parasitic hookworm *Ancylostoma caninum* (Fleming *et al.* 1997, Culetto *et al.* 2004, Kopp *et al.* 2008). However, *Ancylostoma* is closely related to *C.elegans*, and no other research group has to date addressed the role of specific nAChR subunits in levamisole sensitivity and resistance in other nematode clades.

We have shown using a bioinformatics approach that nematodes of clade I and clade III have surprisingly small nAChR gene families compared to the clade V nematode *C.elegans*, a finding which could potentially simplify the task of assigning functional and pharmacological significance to the nAChR subunits from these parasites. We found that sequences encoding 3 proposed components of the levamisole-sensitive nAChR from *C. elegans*, *unc-38*, *unc-29* and *unc-63*, are well conserved between nematodes of different clades. This provides a good basis for more directed studies on the role of these nAChR subunits in levamisole sensitivity in parasitic nematodes from clades I and III, and this work may also allow potential targets to be selected for the design of broad spectrum anthelmintics which will be effective against nematode parasites from all clades.

We have successfully cloned full-length sequences of *unc-38* and *unc-29* from the clade III parasite of pigs *Ascaris suum*, and partial sequence of *unc-63*. The reasons for failing to amplify a full-length sequence of *unc-63* from *Ascaris suum* could not be satisfactorily resolved during the course of the work presented here, though real-time PCR experiments suggest that *unc-63* is expressed at lower levels than *unc-38* and *unc-29* in this nematode. It is expected that the sequencing of the *Ascaris suum* genome, which is currently underway, will in future allow this matter to be explained. The sequences obtained during the course of this work will provide a useful resource for comparing against sequences from any levamisole or pyrantel resistant *Ascaris* isolates as a screen for potential resistance mutations. In addition to this, the ability to

detect these nAChR subunit sequences by real-time PCR will allow any changes in their expression levels in drug resistant *Ascaris* to be assessed, in a similar manner to the work performed by Kopp *et al.*, 2008.

In addition to the amplification of sequence encoding these proposed components of the levamisole-sensitive nAChR, we have amplified a full-length sequence encoding a novel, putatively parasite-specific nAChR subunit not present in *C.elegans*, which has been assigned the name *acr-26*. Work currently being carried out by Hayley Bennett in our laboratory will help to evaluate the potential of this novel receptor as a new anthelmintic target.

Heterologous expression of *Ascaris suum* UNC-38 and UNC-29 in oocytes from *Xenopus laevis* produced functional nAChRs which were subject to two-electrode voltage clamp electrophysiology to elucidate their pharmacology. It was perhaps surprising that just two nAChR subunits would express robustly to produce functional levamisole-sensitive receptors, as UNC-63 is clearly implicated in levamisole sensitivity in other nematode species (Culetto *et al.*, 2004, Kopp *et al.*, 2008). However, this highlights once again the differences which can be found between nematodes of different clades, and cautions against the assumption that *C. elegans* is a valid model for phylogenetically distinct parasitic nematodes. Variations in the responses recorded from oocytes injected with equimolar concentrations of *unc-38:unc-29* RNA, along with an awareness of work performed using the mammalian  $\alpha 4\beta 2$  nAChR, prompted additional experiments to be performed where the receptors produced were deliberately driven to form different stoichiometries using different ratios of *unc-38:unc-29* RNA (Moroni *et al.* 2006). The receptors generated showed a strong resemblance to two of the native *Ascaris* nAChRs: a greater proportion of *unc-38* (presumably giving a 3:2 UNC-38:UNC-29 stoichiometry) produced receptors most sensitive to nicotine and oxantel, matching the N-subtype described from *Ascaris suum* muscle cells, whereas a greater proportion of *unc-29* (presumably giving the alternative 2:3 UNC-38:UNC-29 stoichiometry) produced receptors most sensitive to levamisole and pyrantel, characteristic of the native L-subtype from *Ascaris suum* (Martin *et al.*, 2003; Martin *et al.*, 2004). This is the first time that the phenomenon of alternative stoichiometries displaying different pharmacology has been described for an invertebrate nAChR, and may explain how sufficient signalling complexity can be generated by nematodes with a very small nAChR subunit gene family. We may also speculate that addition of *unc-63* to give a 2:2:1 UNC-38:UNC-

29:UNC-63 stoichiometry would produce the third pharmacological nAChR described from *Ascaris suum*, the B-subtype, defined by buprenorphine sensitivity (Qian *et al.*, 2006). Future work to confirm this hypothesis would require obtaining additional agonist or antagonist compounds which are reported to be specific for the three different pharmacological subtypes, such as the agonists methyridine and buprenorphine, and the antagonists paraherquamide and deoxy paraherquamide (Martin *et al.*, 2003; Robertson *et al.*, 2002). It would also be useful to make concatamer constructs of *unc-38* and *unc-29* where the sequences and resulting subunit proteins are joined together, in order to fix the stoichiometry of the receptors and obtain pure populations of our putative N- and L- subtypes (Ericksen & Boileau, 2007). In the absence of a full length cDNA sequence from *Ascaris suum*, it may be possible to co-express the *Ascaris unc-38* and *unc-29* with the *unc-63* homologue from another nematode species to investigate whether this forms the putative B-subtype. *unc-63* sequences are already available from *Ancylostoma caninum* (Kopp *et al.*, 2008) and *Haemonchus contortus* (Thomas Walsh, unpublished); and the bioinformatics work presented here has already found *unc-63* sequences *in silico* for *Brugia malayi* and *Trichinella spiralis*, which should theoretically make these cDNAs easy to amplify if parasite material for these species could be obtained by our laboratory. It would also be interesting to attempt expression of other parasite nAChRs subunits, in order to determine whether their pharmacology and subunit composition resembles that of *Ascaris* nAChRs, or is more similar to that of *C. elegans*.

In conclusion, the work presented here describes the first study to address the molecular biology underlying cholinergic anthelmintic sensitivity in a clade III nematode, and also describes the first functional expression of a nAChR from any parasite. This provides a sound basis for future work to be undertaken to assess the potential of novel anthelmintics targeting nematode nAChRs in an *in vitro* expression system, and also provides a platform from which the effect of potential resistance mutations in nematode nAChRs can be effectively evaluated.

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## **Appendix i: Primers**

### **SL1**

GGTTTAATTACCCAAGTTTGAG

### **ANCHOR**

(from Boehringer-Mannheim RACE kit)

GACCACGCGTATCGATGTCGAC

### ***Ascaris suum unc-38* homologue**

S1 fw GTCGCGCTTACCGTTTTCTTCC

S2 rv CCATCGCCACATATTTCCAGTCTT

### **5' RACE (rv primers used with SL1 fw primers)**

S72 TAAAGCACGCTGACACCACC

S73 ATAGCACAATATCGGGCACC

### **Full Length**

S84 fw CTGCATTTATTAAGATGTTTGG

S85 rv ATGTAAATTATTGAGTGACTGG

### ***Ascaris suum ASC19452\_1 (acr-26)***

S11 fw TCAAATTCACCCGACACAACATA

S10 fw CGGCAATACGAGAAGGCAGTG

S12 rv AACAAACGCAGACGATTACACG

S9 rv GAACGATGTCGGGCAACCAGAT

### **3' RACE (fw primers used with rv anchor primer)**

3'EST1 TTATCATGGATTTCAAGTGG

3'EST2 TAATTATGTTGTGTCGGGTG

### **5' RACE (rv primers used with fw SL1 primer)**

5'EST3 AACGTTTATCGTCAACACCTG

5'EST2 TCGTCATCGTCATTCCAAGC

5'EST1 TTATCATGGATTTCAAGT

### **Full length**

NewESTall fw ATGATGGCAACTCGTCGG

NewESTall rv TTAATGCAGACCATATAAAGAC

### **Ascaris suum unc-29**

#### **Degenerate primers used initially**

S55 fw ATCAA(CT)GT(AGCT)GA(CT)GA(AG)AA(AG)GA(CT)CA  
S56 fw TGGAC(AGCT)TA(CT)AA(CT)GA(AG)AA(AG)GA(CT)CA  
S57 rv AT(CT)TC(AG)TT(CT)TC(AG)TT(AG)TA(AGCT)GTCCA  
S58 rv CAG(AGCT)GC(AGCT)AC(AG)TA(CT)TTCCA(AG)TC(AG)TC

#### **5' RACE**

S57 rv AT(CT)TC(AG)TT(CT)TC(AG)TT(AG)TA(AGCT)GTCCA

#### **3' RACE**

S65 fw GCACTAAGAGCTATTGACGCG  
S66 fw CATCACTGATCATCTCAAGCAGG  
S67 fw CGAGACGACTGGAAATACATTGC

#### **Full Length**

S82 fw CACTGAGGGCAGTTATGCACC  
S83 rv CAGTGTGGGCGAGATATTAGATC

### **Ascaris suum unc-63 homologue**

#### **Degenerate primers used initially**

S52 fw ATGTG(CT)CA(AG)AT(ATC)GA(CT)GT(ATCG)GA(AG)T  
S53 rv A(AG)(AGT)AT(AG)TCCCA(TC)TCNAC

#### **5' RACE (nested reamplification)**

new5'63a CGACCGTGCCATTTCGACGACA  
new5'63b GACCAAGAACAGTCTCCAAT

#### **Specific Primers for N-terminal**

As63fw AGGATGCCGTGCTTACGCAT  
As63rv TCATGGCGCTTCCCTGGCA

### **Primers with restriction sites for sub-cloning**

For pT7TS:

unc38 BglII fw AGATCTGTTATGTCCGACGTCGCATGCTC  
unc38 SpeI rv ACTAGTTTGAGTGACTGGAGTCGAG  
unc29 BglII fw AGATCTATGCACCTGCCGTCACC  
unc29 SpeI rv ACTAGTTTAGATTTCTCCTCCATT

**Real-time PCR primers**

beta-tubulin fw AACTGCAGTCTGTGACATTCCG  
beta-tubulin rv TGTGAATTCCATCTCGTCCATTCC  
RT unc38 fw GTCTCTTAGAAGCATTGCTATGGCT  
RT unc38 rv GGACATAAAGCACGCTGACACCA  
RT unc29 fw CCTCATCATCGTCTACTATGTGCT  
RT unc29 rv CCACCCTGATGTTCTGAATCT  
RT unc63 fw GTGGATGGTATTGTCCCTGCAGCT  
RT unc63 rv GTCCGTCCAACCTATGTTGCAGCCA

## **Appendix ii: Abbreviations**

AAD	aminoacetonitrile derivative
ACh	acetylcholine
Amp <sup>r</sup>	ampicillin resistance
ATP	adenosine5'-triphosphate
ARS	Ascaris ringer solution
BSA	bovine serum albumin
CPG	controlled pore glass
cDNA	complementary deoxyribonucleic acid
DEPC	diethyl pyrocarbonate
DTT	dithiothreitol
EDTA	ethylenediamine tetra acetic acid
ELISA	enzyme linked immunoabsorbant assay
EST	expressed sequence tag
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GABA	$\gamma$ -aminobutyric acid
GluCl	glutamate-gated chloride channel
HRP	horseradish peroxidase
IgG	immunoglobulin G
IPTG	isopropylthio- $\beta$ -D galactoside
LB	luria broth
LGIC	ligand-gated ion channel
nAChR	nicotinic acetylcholine receptor



NMJ	neuromuscular junction
dNTPs	deoxynucleoside 5'-phosphates
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RACE	rapid amplification of cDNA ends
cRNA	capped RNA
RT	reverse transcriptase
SOS	standard oocyte saline
SL1	splice leader 1
TBE	tris-borate-EDTA buffer
TM	transmembrane domain
TMB	tetramethyl Benzidine
Tris-HCl	tris (hydroxymethyl) methylamine hydrochloride
UTR	untranslated region
X-gal	5-bromo-4-chloro-3-indoyl- $\beta$ -D-galactoside